THE UNIVERSITY OF ALBERTA

ADVANCES IN SMALL BOWEL TRANSPLANTATION:

EFFECTS OF NOVEL IMMUNOSUPPRESSIVE AGENTS ON GROWTH, NUTRITION AND SMALL BOWEL FUNCTION IN NORMAL RATS

by

NATALIE L. YANCHAR, M.D., B.Sc. (Hons.)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

Fall, 1994

ISBN 0-315-95137-0

Canada

THE UNIVERSITY OF ALBERTA

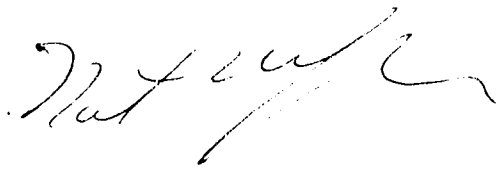
RELEASE FORM

NAME OF AUTHOR: **NATALIE LYNN YANCHAR, M.D., B.Sc. (Hons.)**

TITLE OF THESIS: **ADVANCES IN SMALL BOWEL TRANSPLANTATION: EFFECTS OF NOVEL IMMUNOSUPPRESSIVE AGENTS ON GROWTH, NUTRITION AND SMALL BOWEL FUNCTION IN NORMAL RATS**

DEGREE: **MASTER OF SCIENCE**

YEAR THIS DEGREE GRANTED: **1994**



7207-119 street
Edmonton, Alberta
T6G 1V9

August 29, 1994

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **ADVANCES IN SMALL BOWEL TRANSPLANTATION: EFFECTS OF NOVEL IMMUNOSUPPRESSIVE AGENTS ON GROWTH, NUTRITION AND SMALL BOWEL FUNCTION IN NORMAL RATS** submitted by **NATALIE LYNN YANCHAR** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **EXPERIMENTAL SURGERY**

Supervisor

August 15, 1994

DEDICATION

This thesis is dedicated to my parents, William and Dorothy Yanchar, whose never-ending support and encouragement has guided me throughout my scientific, surgical and research training; but more importantly, has been an inspiration to me throughout my life.

ABSTRACT

The primary obstacle to successful clinical intestinal transplantation is the lack of effective, non-toxic immunosuppressive regimens. This study investigates the effects of cyclosporin (CsA) and the novel immunosuppressive drugs, FK506, rapamycin, mycophenolate mofetil (RS61443), and 15-deoxyspergualin (DSG) on growth, nutrition and small bowel function in normal rats.

Juvenile Lewis rats received 6 weeks of alternate day injections of vehicle (controls), CsA (15 mg/kg), FK506 (2 mg/kg), rapamycin (2 mg/kg), RS61443 (25 mg/kg) or DSG (3 mg/kg). Weight gain, feed intake and animal well-being were monitored. A 3-day balance study, measuring carbohydrate and fat absorption was conducted in the fifth week. *In vivo* intestinal permeability was tested by measuring urinary excretion of orally administered ^{99}Tc-DTPA, lactulose and mannitol. *In vitro* fluxes of 3-O methyl-glucose (3OMeG) and electrophysiological parameters were measured in Ussing chambers and correlated with *in vitro* mitochondrial function studies. Finally, villus morphometry and density were assessed.

FK506, Rapamycin and DSG-treated rats gained significantly less weight than controls and the other groups; the greatest effects were seen in the first two groups. Feed intake was similar in all animals. Anemia, leukopenia, diarrhea and aggressive behavior were also observed in those given FK506 and DSG. Fat and carbohydrate absorption were reduced with CsA, markedly decreased with FK506, increased with RS61443 and unchanged with rapamycin. DSG induced marked fat malabsorption only. Villus hypertrophy was observed in the CsA and RS61443 groups while marked ileal blunting occurred with rapamycin. CsA-treated animals demonstrated increased ileal mucosal-to-serosal (J_{ms}) and serosal-to-mucosal (J_{sm}) 3OMeG fluxes. RS61443 induced a significantly reduced net mucosal-to-serosal (J_{net}) flux in the jejunum, with a compensatory increase in the ileum.. FK506 resulted in the largest changes, with significantly increased J_{ms} and J_{sm} in both regions of small bowel. Conductance, potential difference and intestinal short-circuit current, paralleled these findings and, combined with *in vivo* testing, indicated increased intestinal permeability in all but the RS61443 group. Similar to the 3OMeG fluxes, the most profound permeability changes occurred in the FK506-treated animals. At the cellular level, FK506 and DSG significantly diminished enterocyte mitochondrial energy production.

We conclude that measurable effects on small bowel function are induced by several of these drugs. FK506, Rapamycin and DSG, especially, adversely affect weight gain, nutrient absorption and animal well-being paralleled by marked changes in *in vitro* glucose fluxes, bowel permeability and mitochondrial function. These factors may impact on the utility of these agents as therapy for intestinal transplantation.

ACKNOWLEDGMENTS

The exciting world of transplantation depends on the knowledge, work and dedication of innumerable people. I am proud to have been able to perform these studies, but could not have done so without the great help of many others.

I would like to take this opportunity to thank my supervisor, Dr. David Sigalet, for his unending guidance, encouragement and contagious enthusiasm. His tireless work and efforts in both scientific and clinical surgery are an inspiration. I also wish to acknowledge the helpful advice of my other committee members: the knowledge and understanding of transplant immunopharmacology of Dr. Norman Kneteman, Dr. Garth Warnock's insights on immunology and research design, Dr. Richard Fedorak's knowledge of gastrointestinal physiology and advice on the Ussing chamber and permeability studies, and the guidance of Dr. Ray Rajotte on the technical aspects of experimental design and performance.

Finally, I would like to thank Mr. Gary Martin for his technical assistance in the experiments and Miss Colleen Gardner for her secretarial advice on this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

$\%A_E$	% energy absorption from food eaten
$\%A_F$	% of fat absorption from food eaten
ALG	antilymphocyte globulin
ALS	antilymphocyte sera
APC	antigen-presenting cell
ATG	anti-thymocyte globulin
BN	Brown-Norway
BQR	Brequinar sodium
BUN	blood urea nitrogen
CI	cardiac index
CMC	carboxymethylcellulose
CMV	cytomegalovirus
^{51}Cr-EDTA	^{51}chromium-ethylenediaminetetraacetate
CsA	Cyclosporin A
D.E.	digestible en rgy (kcal)
DSG	Deoxyspergualin
DST	donor-specific transfusion
DSU	donor-specific unresponsiveness
E_A	total daily energy available from diet (kcal/d)
EBV	Epstein Barr virus
E_F	daily energy available from absorption of fat in the diet (kcal/d)
E_G	daily energy available for normal growth in a normal rat (kcal/d)
E_M	daily maintenance energy requirements for a normal rat (kcal/d)

E_w	energy requirement by a normal rat for 1 gram (g) of weight gain
FI	daily feed intake (g/kg/d)
FKBP	FK506-binding protein
g	gram
G	conductance
GALT	gut-associated lymphoid tissue
G-CSF	granulocyte-colony stimulating factor
GFR	glomerular filtration rate
G.I.	gastrointestinal
GM-CSF	granulocyte macrophage-colony stimulating factor
GSF	granulocyte-stimulating factor
GVHD	graft-versus-host disease
HLA	human leukocyte antigen
HPN	home parenteral nutrition
Hsp	heat shock protein
HSV	herpes simplex virus
HVG	host-versus-graft
ICAM-1	intracellular adhesion molecule-1
IEL	intraepithelial lymphocyte
IL-1	interleukin-1
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
IL-3	interleukin-3
IL-4	interleukin-4
INF-γ	interferon-γ

I_{sc}	intestinal short-circuit current
J_{ms}	mucosal-to-serosal flux
J_{net}	net mucosal-to-serosal flux
J_{sm}	serosal-to-mucosal flux
kcal	kilocalorie
kg	kilogram
Lew	Lewis
LFA-1	leukocyte function-associated molecule-1
LP	lamina propria
MAb	monoclonal antibody
MCT	medium-chain triglyceride
M.E.	metabolizable energy
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MLN	mesenteric lymph nodes
MPA	mycophenolic acid
MST	mean survival time
MV	multivisceral
NK	natural killer
NSAID	nonsteroidal anti-inflammatory drug
3OMeG	3-O-methyl-glucose
PD	potential difference
PGI	protaglandin-I
POD	post-operative day
PP	Peyer's patches

PRPP	5-phosphoribosyl-1-pyrophosphate
PTLD	post-transplant lymphoproliferative disorder
R	resistance
R_W	rate of weight gain of a rat (g/d)
RAPA	Rapamycin
RIA	radioimmunoassay
SB	small bowel
SB/L	small bowel/liver
SBS	short bowel syndrome
SBT	small bowel transplantation
SVR	systemic vascular resistance
TCA	tricarboxylic acid
^{99}Tc-DTPA	^{99}Technetium-diethylenetriamine pentaacetic acid
TCR	T-cell receptor
TNF-α	tumor necrosis factor-α
TPN	total parenteral nutrition
TxA_2	thromboxane A_2
V	voltage
W	weight (in kg)

CHAPTER I

INTRODUCTION

Short bowel syndrome (SBS) is a clinical diagnosis characterized by insufficient intestinal function to support the nutritional needs of the patient secondary to massive or repeated resections of small intestine resulting in decreased absorptive surface area (1,2). Until recently, long-term total parenteral nutrition (TPN) has been the only major option for management for many of these patients. However, long-term TPN is fraught with problems, most significantly when used in children (3-5). Catheter thromboses and sepsis, fluid and electrolyte imbalances, micronutrient deficiencies, lifestyle restrictions and high cost can limit the use of this treatment. Hepatobiliary complications are common, and range from abnormal liver enzymes to severe cholestasis and fatal hepatic failure (6-9). Infants and children, especially, are susceptible to problems of cholestasis and liver impairment, the risk being potentiated by concomitant factors such as prematurity and extreme short bowel (10,11). TPN-induced liver failure may be the most common cause of mortality in these patients.

The replacement of nonexistent or non-functioning small bowel with a healthy organ could obviate many of these problems. Although an experimental procedure for decades, only recently has small bowel transplantation (SBT) become a realistic option for patients with SBS (12,13). However, despite advances in technique and periopreative care, there have been relatively few clinical successes thus far, and long-term follow-ups of these patients are not yet available.

Unlike many other vascularized organs, the small intestine is an intensely immunocompetent tissue, presenting unique challenges when considered for transplantation (12). Graft rejection by the host may be complicated by the possibility of graft-versus-host disease (GVHD), although the role of the latter process in SBT is still not completely understood. Despite successes in other organ transplantation, conventional methods of immunosuppression have had limited efficacy in SBT. It is this lack of consistently adequate immunosuppression that has created one of the major stumbling blocks to successful SBT, and has led to

the recent development of a plethora of novel immunosuppressive agents which are currently under investigation for use in transplantation of very immunocompetent vascularized organs such as the small intestine.

However, graft acceptance by the host is not sufficient for the success of SBT. Maintenance of adequate function, including absorption of nutrients and maintenance of an effective barrier from enteric organisms, is imperative. The process of transplantation, itself, is known to have adverse effects on some aspects of small bowel function (16-24). Adverse effects presumed secondary to immunosuppression have also been demonstrated (23,25,26). However, these studies have been performed on transplanted bowel, such that any immunological or mechanical consequences of transplantation may confound the results, making the interpretation of these effects directly attributable to immunosuppression difficult to assess.

Obviously, any further aggravation of function by immunosuppression needs to be minimized. A thorough understanding of the actions of these agents and their effects on growth, nutrition and small bowel function, therefore, needs to be addressed. This is the focus of this thesis.

This thesis will be presented in a paper format. Chapter II is an extensive literature review, briefly discussing the short bowel syndrome, current medical and surgical treatment options and the role of SBT. The unique immunological challenges inherent to SBT will be discussed in the context of methods to circumvent them. Finally, a review of the conventional and novel immunosuppressive agents available, concentrating on their current usage in experimental and clinical SBT will be discussed.

The experimental work undertaken in this Masters of Science in Experimental Surgery and presented in this thesis, sought to examine the effects of some of these immunosuppressive agents on nutrition and small bowel function in normal rats. This is presented in Chapters III through VII. For the sake of practicality, the experiments were run in two sets. Three drugs, cyclosporin A (CsA), rapamycin (RAPA) and RS61443, were studied in the first set of experiments; their results are presented in chapters III through V. The last two agents, FK506 and deoxyspergualin (DSG) were utilized in the second set of

experiments, with their results presented in chapters VI and VII. Finally, chapter VIII is a synthesis of all data obtained, with a discussion of their implications of immunosuppression in SBT.

REFERENCES

1. Wright JK, Jr. Short gut syndrome: Options for management. Compr Ther 1992; 18(8): 5-8.

2. J.M.D. Nightengale JMD, Lennard-Jones JE. The short bowel syndrome: What's new and old. Dig Dis 1993; 11: 12-31.

3. Galea MH, Holiday H, Carachi R, Kapila L. Short-bowel syndrome: A collective review. J. Ped Surg 1992; 27(5): 592-6.

4. Herfindal ET, Berstein LR, Kudzia K, Wong A. Survey of home nutritional support patients. J Parenter Enter Nutr 1989; 13: 255-61.

5. Steiger JU, Srp F. Morbidity and mortality related to home parenteral nutrition in patients with gut failure. Am J Surg 1983; 145: 102-5.

6. Bower RH. Hepatic complications of parenteral nutrition. Semin Liv Dis 1983; 3: 216-24.

7. Sheldon GF, Peterson SR, Sanders R. Hepatic dysfunction during hyperalimentation. Arch Surg 1978; 113: 504-8.

8. Allardyce DB, Salvian AJ, Quenvelle NF. Cholestatic jaundice during total parenteral nutrition. Can J Surg 1978; 21: 332-9.

9. Stanko RT, Nathan G, Mendelow H, Adibi S. Development of hepatic cholestasis and fibrosis in patients with massive loss of intestine supported by prolonged parenteral nutrition. Gastroenterology 1987; 92(1): 197-202.

10. Posthuma R, Trevenen CL. Liver disease in infants, receiving total parenteral nutrition. Pediatrics 1979; 63: 110-5.

11. Touloukian RJ, Seashore JH. Hepatic secretory obstruction with total parenteral nutrition in the infant. J Pediatr Surg 1975; 10: 353-60.

12. Sigalet DL, Kneteman NM, Thomson ABR. Small bowel transplantation: past, present and future. Dig Dis 1992; 10: 258-73.

13. Hiyama DT. The current role of small-bowel transplantation in intestinal failure. Nutr Clin Prac 1993; 8(1): 5-11.

14. Schroeder P, Sandforth F, Deltz E. Glucose absorption after heterotopic small-bowel transplantation. In: Deltz E, editor. Small Bowel Transplantation. Experimental and Clinical Fundamentals. Berlin: Springer, 1986: 74-8.

15. Schraut WH, Lee KKW, Sitrin M. Recipient growth and nutritional status following transplantation of segmental small-bowel allografts. J Surg Res 1987; 43: 1-9.

16. Schroeder P, Deltz E, Seifert J, Sandforth F, Thiede A. Absorptive capacity of the transplanted small bowel. Gut 1987; 28 (suppl 1): 275-9.

17. Watson AJM, Lear PA, Montgomery A, Elliot E, Dacre J, Farthing MJG, Wood RFM. Water, electrolyte, glucose, and glycine absorption in rat small intestinal transplants. Gastroenterology 1988; 94: 863-9.

18. Raju S, Fujiwara H, Grogan JB, Achord JL. Long-term nutritional function of orthotopic small bowel autotransplants. J Surg Res 1989; 46: 142-6.

19. Sigalet DL, Kneteman NM, Fedorak RN, Kizilisik AT, Thomson ABR. Intestinal function following allogeneic small intestinal transplantation in the rat. Transplantation 1992; 53(2): 264-71.

20. Quigley EMM, Thompson JS, Rose SG. The long-term function of canine jejunoileal autotransplants: Insights into allograft physiology. Transplant Proc 1992; 24(3): 1105-6.

21. Ishii H, Hashimoto N, Kitada T, Kuroki T, Utsnomiya J. Malabsorption after small-bowel transplantation. Hepato-Gastroenterol 1993; 40: 282-4.

22. Lee KK, Hurst RD, Schraut WH. Enteral Cyclosporin A absorption following orthotopic small-bowel transplantation. Gastroenterology 1987; 92: 1497.

23. Sigalet DL, Kneteman NM, Fedorak RN, Kizilisik T, Madsen KE, Thomson ABR. Small intestinal function following syngeneic transplantation in the rat. 1993; Submitted.

24. Ballinger WF, Christy, MG, Ashby, WB. Autotransplantation of the small intestine: The effects of denervation. Surgery 1962; 52: 151.

25. Collin J, Dennison AR, Watkins RM, Millard PR, Morris PJ. Segmental small intestinal allografts: II. Inadequate function with Cyclosporine immunosuppression: Evidence of a protein-losing enteropathy. Transplantation 1987; 44(4): 479-83.

26. Greenstein SM,Sun SC ,Schechner RS ,Senitzer D ,Tellis VA. Prolonged use of cyclosporine (CsA) produces reversible hemodynamic and absorptivr aslterations in the transplanted rat small intestine. In: Abstracts of the 27th annual meeting of The Association for Academic Surgery; 1993 Nov 10-13: abst #33.

CHAPTER II

THE SHORT BOWEL SYNDROME AND THE ROLE OF SMALL INTESTINAL TRANSPLANTATION: CLINICAL ASPECTS, IMMUNOLOGY AND IMMUNOMODULATION

THE SHORT BOWEL SYNDROME

DEFINITION AND ETIOLOGY

The short bowel syndrome (SBS) is a clinical diagnosis characterized by insufficient intestinal function to support the nutritional needs of the patient and maintain an adequate quality of life (1). This may be the result of massive loss or resection of small bowel or a diffuse pathophysiological process impairing its global function. In adults, the cause of SBS is most often secondary to massive enteric resection due to catastrophic mesenteric vascular occlusion, inflammatory bowel disease, midgut volvulus, tumor, trauma and radiation enteritis (1-3). Interestingly, this has changed since the early decades of this century, when strangulated hernias and volvulus were the most commonly reported etiologies (4,5). In the pediatric age group, causes include resection for volvulus due to malrotation or around an intact vitelline duct, necrotizing enterocolitis, congenital atresia, gastroschisis or strangulated hernias (1-3).

The development of intestinal insufficiency is related not only to the overall length of remaining bowel but also to which portions of intestine are preserved and their functional and absorptive capacities. It is generally recognized that massive loss of the proximal small bowel is better tolerated than that of the distal small intestine. This is due to the ileum's absorptive function of bile salts and vitamin B12, its slower transit rate, and its better ability to morphologically and functionally adapt after resection (2,6,7). As well, the terminal ileum can respond to the presence of unabsorbed nutrients, especially fat, by slowing proximal jejunal transit, mediated by gut hormones (8,9,10). This 'ileal brake' mechanism as well as the

ileocecal valve permit the bowel the ability to control the rate of intestinal transit and therefore control intestinal absorption. By delaying the passage of luminal contents, it increases time for absorption, and also prevents bacteria from entering the small bowel from the colon where subsequent colonization would accentuate malabsorption of nutrients, water and electrolytes (11,12). Finally, resection of the colon aggravates loss of water and electrolytes, worsening the prognosis (13).

Several authors have attempted to define the minimum length of bowel required to maintain long-term survival in patients with SBS. Wilmore found that infants who were left with less than 40 cm of small intestine after a small bowel and ileocecal valve resection did not survive (14). However, long-term survival was possible with at least 15 cm of small bowel, provided that the ileocecal valve and colon were still present. Similar results have been reported by others with successful adaptation to total enteral nutrition in infants and children with 13 to 20 cm of small bowel in the presence of an ileocecal valve (15-18).

These limits have been extended. With better overall care of these patients, there have been reports of neonates able to adapt with as little as 8-15 cm of small intestine with an ileocecal valve (15,19-23). In addition, unlike Wilmore's findings, children have survived up to 7 years with no ileocecal valve and as little as 15 cm of jejunum, albeit dependent on parenteral nutrition (24).

The presence of an ileocecal valve is not mandatory for intestinal adaptation with very short bowel (25). Caniano reported one child with only 20 cm of small bowel, the left hemicolon and no ileocecal valve who was able to adapt to total enteral nutrition by 33 months of age. At reoperation for restoration of intestinal continuity, re-measurement of the small bowel revealed a significant increase in length to 45 cm (26). A similar case was reported by Weber *et al* of a child with a massive intestinal resection including the ileocecal valve leaving only 22 cm of small bowel (27). Nutritional independence with a normal diet was attained within 28 months.

Obviously, adaptation of the remaining gut is vital to the outcome of SBS. Unfortunately, however, our ability to manipulate this process to improve clinical outcome is limited.

Extensive reviews on intestinal adaptation in SBS have been published (2,3,7,28). Two basic changes occur in this process: structural and functional.

Increasing the mucosal surface area available for nutrient absorption occurs on a gross level with dilatation and lengthening of the residual bowel as just described, and at a cellular level through increases in crypt depth and cell number, resulting in an increase in villus length (2,15,26,27,29). These compensatory responses have the capacity to increase the remaining small intestinal surface area up to 4-fold (1,3). Functionally, the qualitative absorptive processes of individual enterocytes in their ability to transport amino acids and glucose is unaltered (3,7). However, the density of these carriers per villus may increase as has been observed by Fedorak *et al* and others with the glucose carrier in response to the presence of an increased concentration of substrate (30,31). As well, alterations in tight junction permeability can enhance glucose absorption through the paracellular route in the presence of increase levels of glucose, although the quantitative importance of this process to functional adaptation has been questioned (31,32).

The signals involved in adaptation and the processes by which this occurs are still not fully understood. In general, adaptation appears to be related to the increased concentration of unabsorbed nutrients and pancreaticobiliary secretions at the brush-border membrane and/or a long-list of hormonal factors which may increase during the adaptive process, such as enteroglucagon, secretin, cholecystokinin and gastrin (3,7,33).

PROBLEMS OF THE SHORT BOWEL SYNDROME

Significant problems can arise in patients with SBS which can be categorized as: a) fluid and electrolyte, b) metabolic, c) nutritional and growth and d) TPN-related (1,2). These can affect all patients with SBS, but the last category tends to be the most vital for the pediatric age group as it strongly contributes to morbidity and mortality (6,18,26,27).

Fluid, Electrolyte, Metabolic & Nutritional Problems

A major problem encountered in patients with SBS is massive loss of fluids and electrolytes (notably sodium and magnesium) (2,3). This is most prominent in those with a jejunostomy, because of loss of the absorptive capacity of the distal small bowel to handle the 8-10 liters of intestinal fluid produced by daily gastric, pancreatic and biliary secretions (1,34). Dehydration and electrolyte imbalances can occur rapidly. Further aggravation occurs with any loss of duodenal and proximal jejunal inhibitory neurohormonal controls, subsequently leading to gastric hypersecretion. The subsequent decreased pH of secretions also induces pancreatic enzyme inactivation, resulting in protein and lipid maldigestion and malabsorption.

Loss of the terminal ileum interrupts the enterohepatic circulation of bile salts and contributes to malabsorption of fat and the fat soluble vitamins, A, D, E and K. It also results in bile salt diarrhea and steatorrhea (1). Bile salts in the colon subsequently stimulate the absorption of small molecules such as oxalate, resulting in hyperoxaluria and oxalate renal stones (35). In addition, undigested fats chelate Ca^{2+} and Mg^{2+}, causing their malabsorption; intestinal oxalate, normally rendered insoluble by combining with Ca^{2+}, subsequently remains soluble in chyme, enhancing its absorption in the colon (1). Finally, chronic dehydration further aggravates this tendency to nephrolithiasis and the development of oxalate stones (2).

Added to these effects is the overall decreased mucosal area for absorption, allowing partially digested polysaccharides, proteins and fats to enter the colon. This promotes bacterial overgrowth, leading to the production of short-chain fatty acids and lactic acid. These aggravate the osmotic diarrhea, while subsequently produced gas may lead to severe bloating (3).

TPN-Related Problems

With advances in medical management of SBS and parenteral nutrition formulations, most of the metabolic and nutritional problems of SBS can be well-controlled, whereas TPN-related complications can frequently be fatal (for excellent reviews, see references 1 and 2). In the broadest sense, these can be grouped into lifestyle, catheter-related, metabolic and hepatobiliary.

Lifestyle, Emotional and Economic Consequences of TPN: Because of their total dependence on parenteral nutrition, these patients have significant lifestyle restrictions. In addition, they may develop feelings of dependency as well as other psycho-social consequences (36-39). Frequent depression and episodes of anxiety over catheter use and problems are not uncommon.

The cost of long-term TPN can be substantial (36,40). As published in several reviews, the annual cost in the USA ranges from $60,000 to $150,000 per year (6,41,42). In our province, there is an estimated 40 patients on home parenteral nutrition (HPN): 35 adults, most with SBS, and 5 children, all with SBS, with an estimated cost per patient of $110 (Canadian) per day or $30,200 (U.S.) per year (Marriage B. Home TPN Coordinator, University of Alberta Hospitals, November, 1993.). There are also significant added expenses of repeat hospitalizations for HPN-related complications. Indeed, adult HPN patients can average two hospitalizations per year, with at least one episode secondary to parenteral nutrition-related complications, usually catheter-related sepsis. Pediatric HPN patients average four hospitalizations per year, with 2.5 of these being related to parenteral nutrition (36).

Catheter-related Problems of TPN: Catheter-related complications are the most common problems encountered in patients on long-term TPN (38). Bacterial contamination of the catheter can lead to septicemia which may become rapidly fatal. Recurrent thromboses of catheter sites frequently leads to multiple venous access procedures with eventual exhaustion of available access sites. In a recent review of SBS patients in the U.K., catheter sepsis was the major source of in-hospital morbidity, seen in 54% of patients; 30% died from sepsis (43). Another large series of HPN patients revealed that 60% of hospital admissions were secondary to catheter-related complications including sepsis, catheter clotting, site infection and positional problems (44).

Metabolic Problems with TPN: Fluid and electrolyte imbalances and micronutrient deficiencies can occur with TPN. However, advances in formulations and careful individual monitoring has made these problems relatively uncommon. Altered bone metabolism results in an incidence of metabolic bone disease in patients on TPN as high as 15% (44). The exact pathogenesis of this is unknown but is felt to

be related to abnormal metabolism of calcium, protein and vitamin D, as well as aluminum contamination of TPN solutions (45,46). This can result in osteomalacia with debilitating bone and joint pain, vertebral body compression and pathologic fractures. Withdrawal of TPN will usually cause this syndrome to subside, however, this is not an option in those patients with irreversible intestinal failure who are otherwise dependent on TPN for life.

Hepatobiliary Complications of TPN: Prolonged use of TPN can result in a wide range of hepatobiliary abnormalities ranging from abnormal liver enzymes in almost all patients, to severe cholestasis and fatal hepatic failure (47-51).

Steatosis (increased accumulation of fat in the liver or "fatty liver") is the most common morphologic change associated with TPN in adults (51,52). In non-cirrhotic adult patients, this may occur as frequently as 55% to 75% of the time (53-55). Clinically, this is most frequently manifested by benign and reversible elevations in "liver function tests". Increased transaminase levels are the most common, onsetting between 1 and 2 weeks after starting TPN in adults. Elevations in alkaline phosphatase and bilirubin are not as prevalent, but may occur approximately 2 to 3 weeks after starting TPN (53,54,56,57). This is felt to be due to the infusion of large amounts of glucose, which is hypothesized to stimulate hepatic acetyl-CoA carboxylase activity, the rate-limiting step in fatty acid synthesis, thereby increasing the lipid content of the liver (54,58-60). When part of the glucose calories are replaced by a fat emulsion, such elevations in liver enzymes are not observed (58,61).

Intrahepatic cholestasis becomes evident after about 2 to 3 weeks of TPN and can be seen in up to 50-75% of patients (57). The pathogenesis of this is likely multifactorial, related to the patient's clinical condition and nutritional status, bacterial overgrowth in the remaining small bowel, metabolism of unabsorbed bile acids by colonic organisms and/or hepatotoxicity of the TPN formula itself (52). Some studies have shown a correlation between the amount of lipid infused and the development of biochemical and histopathological cholestasis (57,62).

Progression to chronic liver disease is reportedly infrequent within the adult population on long-term TPN, and is strongly associated with long-term administration (53,54). One series revealed a 10% incidence of clinically severe liver disease; two of these 6 patients (3% overall) died of liver failure (63). Again, the mechanism of this is likely multifactorial, related to the primary disease, clinical and nutritional status of the patient and hepatotoxicity of the TPN formulations (52). It would appear that massive loss of intestine also contributes to the development of liver disease. Stanko *et al* investigated 18 patients on TPN for at least one year and found that 4 of the 6 patients who had undergone a massive intestinal resection (from the ligament of Treitz to the mid-transverse colon) developed severe cholestasis with bile ductule proliferation, portal and periportal inflammation and mild steatosis; two of these patients also had significant hepatic fibrosis (64). Patients who had no or only modest loss of intestine (30 to 100 cm of small and/or large bowel) showed no evidence of hepatic dysfunction over a period of one year on TPN.

The problems of cholestasis and liver impairment are accentuated in infants and children (18,51). An estimated 30 to 40% of all infants on TPN may eventually develop severe cholestasis, with the development of pathologic changes of portal fibrosis and cirrhosis (65,47). More significantly, even those infants who survive TPN-induced hepatic dysfunction, to be eventually weaned to enteral nutrition, may have persistent portal fibrosis and cirrhosis found on liver biopsy, suggesting the persistent risk of chronic liver disease despite amelioration of clinical cholestasis (65).

Infants, especially premature, have added risk factors for liver disease which can complicate TPN-induced cholestasis, such as an immature liver enzyme system, sepsis and longer duration requirements for TPN (50,51). Prolonged fasting with its associated decrease in hormonal and neural stimulation of hepatic bile secretion (66), coexistent disease processes such as sepsis, and intestinal stasis and colonization, resulting in interruption of the enterohepatic circulation of bile salts (51), further aggravate this process. Indeed, the incidence of TPN-induced hepatic dysfunction in this premature population may be as high as 84% (51,67). Composition of the TPN solutions, themselves, may play a role in this

pathogenesis. Large volumes of amino acids in the infusates (68,69), inadequate levels of methyl donor amino acids such as serine (70), and deficient amounts of taurine (the principle bile acid conjugate in neonates which promotes bile flow and protects against lithocholate toxicity) (71,72) have all been implicated.

This risk is even further increased in those with a short gut (19,73). Progression to liver failure is, unfortunately, not uncommon in this young age group. In one series , death from TPN-induced liver failure occurred in 5 of 60 infants and children with SBS, making it the most common cause of mortality (18).

OUTCOMES OF PATIENTS WITH SHORT BOWEL SYNDROME

Early mortality in patients with SBS, as the result of dehydration, sepsis and malnutrition, is now relatively uncommon (6,18,26,27). However, over the longer term, 3-year survival rates range from only 65% to 80% for those on HPN. In adults, mortality is generally related to the underlying disease process with mortality rates over the first few years of treatment ranging from 5% (Crohn's disease) to 20% (ischemic bowel disease, radiation enteritis, chronic motility disorders), eventually stabling out to an annual rate of 2% to 5% respectively. Deaths directly related to TPN in this adult population are uncommon (36,38).

In the pediatric age group, large series report survival rates of 85 to 95% over 2 to 10 years, considering all cases of SBS based on a bowel length of 50% of "expected" length or less than 100 cm (18,27). Deaths in these cases are related to either complications of prematurity (sepsis, bronchopulmonary dysplasia) or to complications of TPN (most often, liver failure). In cases of extensive SBS, however, the prognosis is much more guarded. A review by Hancock *et al* of infants with 16 cm or less of small bowel resulted in a 100% mortality rate within 8 months, despite optimal medical and surgical management (73). Three of the infants (43%) died from TPN-related progressive liver failure.

For those children (and adults) with SBS, whose remaining bowel fails to fully adapt, what are the options? In the past, a uniformly poor prognosis was expected, based on nutritional deficiencies and complications of parenteral feeding. However, significant improvements in TPN and overall care, and the development of intestinal lengthening procedures have resulted in a more optimistic outlook for these patients (25,34). More recently, however, the evolution of small bowel transplantation (SBT) has led to hope for an eventual "cure" for SBS.

MANAGEMENT OF SHORT BOWEL SYNDROME

Medical Management

The medical management of SBS aims at reducing and ameliorating the effects of high output, micronutrient deficiencies and other metabolic complications (2,3). As discussed above, a major problem of patients with short bowel, especially those with jejunostomies, is a high intestinal output of water and electrolytes, especially sodium and magnesium. For those patients able to handle some oral intake, oral replacement solutions are available. When these losses are excessive (greater than 2 liters daily), parenteral fluids are required. Magnesium supplements may also be needed. Reduction of intestinal motility with codeine phosphate and loperamide aid in reducing high outputs, by decreasing intestinal motility and through antisecretory actions. Further reductions of gastric acid and intestinal output can be achieved with H_2 blockers, omeprazole or octreotide.

For those patients taking part of their nutrition enterally, high energy and protein-containing foods are advised (74,75). Extra fat is advantageous as it supplies a good energy source that does not increase the osmotic load (75). Lactase deficiencies may occur in those with extensive loss of small intestine and worsen diarrhea; in these patients, a lactose-free diet is recommended. Continuous or nocturnal enteral feeding may be given in addition to a normal diet, with the advantage of providing nutrients to the gut at a slow rate, allowing maximal use of residual intestine. Providing maximal hydration, a low oxalate diet and substances which bind oxalic acid in the colon, such as cholestyramine, may help prevent the development of oxalate nephrolithiasis.

Patients who cannot tolerate enteral feeding have benefitted from the numerous advances made in parenteral nutrition, with the development of protein hydrolysates, hypertonic glucose solutions and fat emulsions. Central venous cannulation is required to allow the delivery of these substances, and meticulous care must be taken to prevent the complications of catheter sepsis and thrombosis. Vitamin B_{12}, iron, zinc, vitamin D and essential fatty acid deficiencies may occur and must be supplemented.

Surgical Management

Several investigators have looked at surgical procedures in an attempt to increase the absorptive capacity of any remaining small intestine in these patients. These have been extensively reviewed by Thomson (76,77), Warner *et al* (78) and Nightengale *et al* (2).

Aims to slow transit have looked at interpositioning of antiperistaltic segments of bowel. Indeed, small bowel segments of 10 cm in adults and 3 cm in children have been shown to improve absorption of water and salts, and ameliorate diarrhea in SBS, as have interposed segments of colon in infants and children (79-83). However, these methods are limited by inconsistent results, complications such as intestinal obstructive symptoms and the fact that many patients with very extreme SBS may not be able to afford to sacrifice a segment of intestine for reversal (77). In addition, they do not actually increase the surface area available for nutrient absorption--the primary problem of SBS.

Several attempts to create intestinal valves and sphincters in order to lengthen transit time and prevent retrograde reflux of colonic contents have involved constricting the intestine externally, denervating segments of intestine surgically or chemically, and intussuscepting intestinal segments to create a valve-effect (77,84-89). These procedures have been generally limited, however, by technical complications such as obstruction, necrosis of the valve-construct and intussusception.(77).

As well as decreasing transit time, improvement of the function of existing intestine has been investigated. It is not uncommon for the proximal small bowel to become markedly dilated secondary to chronic obstruction and adaptation in SBS, resulting in inefficient peristaltic action and poor propulsion

of intestinal contents. Subsequently, there is also an increased risk of bacterial overgrowth in the dilated segments. Surgical tapering of the bowel by resecting or imbricating redundant tissue along the longitudinal axis has been shown to improve intestinal function and absorption in these patients (90,91). As an extension of tapering, intestinal lengthening procedures, utilizing the redundant tissue, have evolved (92-94). Commonly known as a "Bianchi procedeure", the dilated bowel is transected longitudinally, and the resulting two segments anastamosed end-to-end to double the length of the bowel. Several recent reviews have reported 77% to 100% success rates with this procedure in completely or partially converting TPN-dependent infants and children with SBS to enteral nutrition (77,95-97). However, this procedure is not without its problems; complications such as the development of entero-enteric fistulas, and dilatation of the "Bianchied" region with obstructive symptoms have been reported (98).

More recently, the Iowa model of bowel elongation has been described. This procedure involves inducing the antimesenteric side of the small bowel loop to parasitize the vascular supply of other host abdominal organs followed by longitudinally splitting the bowel. This provides two loops with mesenteric and antimesenteric blood supplies, respectively, which can be anastamosed together, end-to-end. Developed by Kimura *et al*, this procedure has been used with some success (99-103).

Experimentally, growth of new intestinal mucosa has been investigated by patching small bowel defects with surfaces such as colonic serosa, abdominal wall pedicles, human amniotic membrane and prosthetic materials, with an aim to allow for ingrowth of the neomucosa (77,104-108). Some successes have been achieved in animal models of SBS (107,109,110). However, the process is restricted by factors such as the size and location of the defect replaced, the patching surface used, growth factors and inconsistent results. Surgical complications such as post-operative obstruction and infected prosthetic materials are common. No clinical experiences with this procedure have been reported.

SMALL BOWEL TRANSPLANTATION AND ITS ROLE IN TREATMENT OF THE SHORT BOWEL SYNDROME

The best method of treating SBS in any age group would be the replacement of nonexistent or non-functional small intestine with a healthy one. However, the risks and benefits of small bowel transplantation (SBT) must be carefully weighed against those of present therapeutic options. For SBT to be justified, the patient must have definitive irreversible gut failure and permanent dependence on TPN. Their overall clinical condition and life expectancy must be expected to improve significantly as compared to management with permanent HPN. Obviously, children, who tend to develop TPN-related complications more frequently than adults and who have a greater number of potential life years, are stronger candidates for SBT. Several centers have looked at their SBS population to determine the number of SBT candidates expected. Amongst the adult population, estimates of 12-40% of patients with SBS have been quoted (111). In North America, approximately 1 to 2 per million people would benefit from SBT annually (112). Estimates of extreme SBS among neonates range from 0.3 to 0.5 per 10,000 births per year. The majority of these will not adapt to enteral nutrition and would also become candidates for SBT (113).

On a financial level, the cost of SBT has been estimated at $500,000 per transplant plus $20,000 per year for medical follow-ups and immunosuppressive medications (114). In addition, the costs of rehospitalizations for episodes of rejection and treatments for potential adverse effects of long-term immunosuppression must be considered. As a comparison to the costs of long-term TPN (discussed above), not enough clinical experience has been accrued to determine whether SBT is a long-term cost effective alternative for management of SBS.

Ideally, SBT should be considered for those patients who, at the time of development or diagnosis of SBS, will obviously not adapt their remaining small bowel to allow for enteral nutrition, and will develop permanent complete dependence on TPN. It should be performed early, before the metabolic and physiologic complications of long-term TPN develop. At the present time, however, SBT is still in the

experimental stages and therefore can only be advocated as a "last resort" for patient who can no longer be sustained with more conservative treatments presently available.

THE HISTORICAL DEVELOPMENT OF SBT

Excellent reviews on the historical development of experimental and clinical SBT have been published (6,41,42,115,116). However, it was not until the development of cyclosporin A that the potential success of SBT became a reality. A hallmark study by Reznik *et al*, in Toronto, subsequently demonstrated that cyclosporin A (CsA) was able to prevent, or at least delay, reject on the canine model (117). Since then, a plethora of experimental work has been done to expand on the use of CsA, develop new immunosuppressives and further understand the unique, yet often frustrating, immunological consequences of SBT.

The history of SBT dates back to the early part of this century when Alexis Carrel demonstrated the technical feasibility of SBT in dogs (118). Enthusiasm for transplantation of vascularized organs dwindled after that because of problems with immunosuppression and overwhelming rejection. Lillehei *et al* pioneered further work in the late 1950's, and were amongst the first to publish a clinical case of SBT in 1967 (119). Theirs, was a 46-year old woman who underwent a massive small and large bowel resection secondary to infarction. She subsequently received the entire small bowel from a cadaveric male donor but, unfortunately, died very soon post-operatively from multiple pulmonary emboli. At autopsy, however, the vascular anastomoses of the bowel and the graft itself appeared to be viable. Thus, very early on, the technical feasibility of SBT in humans was demonstrated.

Since then, scattered reports of clinical attempts at SBT had been reported, but without adequate immunosuppression, the same difficulties of Carrel were encountered: unequivocal rejection of the graft, frequently resulting in a fatal outcome of the host (115,120-122). However, over the past few years, some successes have been achieved, although followups are relatively short at this time.

THE CURRENT STATUS OF CLINICAL SBT

The first successful isolated small bowel transplant recipient recieved a neonateal allograft in Paris under CsA immunosuppression, with only three bouts of mild rejection within the first 19 months, successfully treated with anti-interleukin-2 (IL-2)-receptor monoclonal antibodies or anti-thymocyte globulin (116,123,124). She was continuing to grow and develop normally on a totally enteral diet while still maintained on CsA at last report, 4 1/2 years later (125). The first successful combined small bowel-liver transplant was performed in London, Canada, also under CsA immunosuppression (126). Only one mild episode of graft rejection occured, at 9 weeks post-transplantation, which responded well to OKT3. She was also doing well at last report, 5 years later (127).

To date, the largest series have been published by Todo *et al* in Pittsburgh (128-133). The most recent was a 3 1/2 year accumulation of 61 cases utilizing a new immunosuppressive agent, FK506, with rejection episodes treated with increased FK506 doses, steroid boluses or OKT3 (133). Twenty-two patients (15 adults, 7 children) have received an isolated intestinal graft (SB), 26 patients (6 adults, 20 children) received a combined small bowel-liver graft (SB/L), 11 (6 adults, 5 children) received a multivisceral organ grafts (MV) and two cases were unsuccessful. Minimum follow-up was 2 months. One year graft survival rates were 70%, 73% and 80% for the SB, SB/L and MV grafts. However, overall, the 24 month graft survival has been only 53%. Fatal complications included acute rejection with sepsis, frequently worsened by cytomegalovirus (CMV) infection, sepsis secondary to technical failures or TPN catheter infections, pneumonia and the development of post-transplantation-lymphoproliferative disorder (PTLD) (1 patient). These resulted in 5 deaths in the SB group (23%), 10 in the SB/L group (38%) and 2 in the MV group (18%). Functionally, 34 patients are TPN-free (66%), and 4 are on partial TPN only (7%).

Smaller series have been published out of London, Canada (127), the United Kingdom (134) Nebraska (135) and Chicago (136). Taken together, they report 15 cases, 2 of which have received either an isolated SB allograft, 11 a SB/L allograft, and 4 a MV transplant, all under CsA or FK506

immunosuppression. To date, one of the isolated SB transplants is alive and well at 11 months under FK506 immunosuppression (134); the other required graft removal at 2 weeks because of rejection under CsA (127). Seven patients recieving a combined SB/L allograft are doing well at follow-ups of a few weeks to 5 years, 3 under CsA immunosuppression and 4 receiving FK506 (127,135). One patient has died from rejection on CsA (135), 2 from sepsis (127,135) and one has developed PTLD while on FK506 (134). Of the 4 patients receiving MV grafts with CsA immunosuppression, one is reportedly well at 5 months (136), while the other 3 have succumbed to PTLD (127,136).

Obviously, the success of SBT is still limited by the lack of adequate immunosuppression protocols Rejection secondary to undersuppression or the development of sepsis of PTLD due to oversuppression are the major causes of morbidity and mortality in SBT recipients. Why then, is transplantation of the small intestine such an immunological challenge?

IMMUNOLOGICAL CONSIDERATIONS WITH SBT

The small bowel is unique amongst vascularized organs in that it contains a large amount of immunocompetent cells within the mesenteric lymph nodes (MLN), and the gut-associated-lymphoid tissue (GALT), which includes the Peyer's patches (PP), lamina propria (LP) and intraepithelial lymphocyte (IEL) compartment. The exact roles of the MLN and GALT in the immunological interplays, within SBT, have been under much investigation. These cells become activated when transplanted into a non-major histocompatibility complex (MHC)-identical recipient and direct themselves against cell surface antigens of host epidermal cells. Subsequently, the phenomenon of graft-versus-host-disease (GVHD) as well as the more commonly seen host-versus-graft (HVG) rejection response can potentially occur (41,137).

Before describing these responses in specific relation to SBT, the basic concepts and processes of HVG rejection and GVHD should be briefly reviewed. The importance of these concepts will become more apparent when discussing immunomodulatory efforts to circumvent them. Obviously, these

responses are complex and still under much investigation, and so this discussion is by no way complete. The reader is subsequently referred to more detailed reviews available (138-140).

Foreign antigen of the donor cells of an allograft can be recognized directly by host $CD8^+$ ($T_{cytotoxic}$ or T_c) cells, binding to MHC class I antigen by their T-cell receptor (TCR) or indirectly when the TCR on $CD4^+$ (T_{helper} or T_h) cells bind to processed donor MHC antigen on the surface of host antigen-presenting cells (APC). Adhesion molecules on the surface of both T-cells (i.e. leukocyte function-associated molecule-1 or LFA-1) and donor epithelial cells (i.e. intracellular adhesion molecule-1 or ICAM-1) can promote this interaction as well as transduce regulatory signals to the T-cells (141-143). In addition, B-cell activation can occur in response to antigen binding to surface IgM to set off the humoral components of the immune response. Secondary or co-stimulatory signals are required and provided by cytokines, released by the APCs and activated T-cells. Release of interleukin-1 (IL-1) by APCs further activates T_h cells, release of IL-2 by activated $CD4^+$ cells further stimulates T_c cells while B-cells require the release of IL-4 from these cells. Just as important, autocrine loops serve to amplify these reactions, such as the up-regulation of IL-2R by activated T_c cell precursors, which enhances their responsiveness to IL-2 to allow them to differentiate and mature into powerful T_c cells. Synthesis and release of this wide array of cytokines, also including IL-5, IL-6, IL-10, tumor necrosis factors (TNF)-α and β, granulocyte macrophage-colony stimulating factor (GM-CSF), and interferon (IFN)-γ, all with overlapping actions on cells of different lineages, results in the activation, proliferation and differentiation of T- and B-cells, macrophages and other hematopoietic elements into cells with specific and diverse roles. These processes are outlined in Figure II-1.

This antigen-specific cellular response in the graft results in the recruitment and activation of nonspecific cells to produce a marked inflammatory response as well as the development of effector functions of specific T-cell natural killer (NK) or macrophage-mediated cytotoxicty, culminating in graft rejection and donor cell death.

With the strong immunogenicity of the small bowel, as discussed above, these responses can essentially work 'in reverse' whereby the transplanted lymphocytes react against the 'foreign' antigens of the host and GVHD ensues. In an immuno-incompentent recipient, such as seen with bone marrow transplantation, where GVHD has been typically characterized, the response can be intense and frequently fatal. In an immunocompetent recipient, however, where the host can react against these transplanted lymphocytes, the response may be less severe; indeed, the complete role of GVHD in SBT has been questioned as will be discussed below.

The Roles of Host-Versus-Graft Rejection and Graft-Versus-Host Disease

In order to study the responses of HVG rejection and GVHD more fully, Monchik and Russel devised an excellent rat model which has become the basis of most such investigations today (144). Upon mating two non-MHC identical rat strains, Lewis (Lew) and Brown-Norway (BN), an F_1 hybrid is developed. When the $LBNF_1$ is used as a donor into one of the parenteral strains, the host recognizes part of the transplanted bowel as foreign and rejects the graft. When the $LBNF_1$ is the recipient of a graft from one of the parental strains, the graft recognizes certain MHC characteristics of the host as foreign and the phenomenon of GVHD ensues. In the fully allogeneic transplantation model (BN $\rightarrow$ Lew and Lew $\rightarrow$ BN), however, only HVG is seen clinically and histologically (144).

At a cellular level, the roles of the MLN and GALT in these reactions has been under intense study. An important observation is the rapid migration of donor graft-associated lymphocytes into the host's reticuloendothelial system and the reverse transit of host lymphocytes into the graft lymphoid tissue, also known as "two-way trafficking" of immunocompetent cells . Almost immediately after transplantation, donor lymphocytes can be isolated from the host's circulation, and this has been seen to persist up to 12 to 54 days in clinical cases (under FK506 immunosuppression) (145). More intriguing, perhaps, is the repopulation of donor lymphoid compartments by host cells. Following SBT in the rat model, graft MLN and PP become heavily infiltrated with host cells within the first post-operative day (POD 1). Mucosal infiltration is somewhat slower and not substantial until POD 5 (146). This is reflected clinically, where

functioning and non-rejecting small bowel allografts are associated with complete lymphoid repopulation of the graft within 10 to 12 weeks (145). It would therefore appear that the presence of recipient lymphocytes in the graft does not imply rejection, but is an indication of graft acceptance by the host.

In the one-way rejection model ($LBNF_1 \rightarrow$Lew), graft rejection occurs within 6 to 9 days. The progression of this response has been well described (144,147-149). Histopathologically, it is felt to onset by an initial attack on the vascular endothelium and crypt epithelium of the graft. By POD 3, slight mucosal damage, restricted to the crypts, is seen with an associated progressive obliteration of the mural microvasculature. This onsets within the first 24 hours post-transplant, with evident lesions of the arterioles and venules at the junction of the mucosa and submucosa by POD 3 (150). By POD 6, the crypts have undergone extensive cell damage, the lamina propria is distended with a mononuclear cell infiltrate, and endothelial cell injury of the entire mural vasculature is evident. This marks phase I of the rejection response. Phase II is evident by POD 9 to 10, with gross villous blunting, scattered epithelial sloughing and more intense cellular infiltration. At the end of complete rejection by POD 10 to 13, heavy transmural infiltration by lymphocytes, plasma cells and polymorphonuclear white cells marks complete mucosal destruction. By the completion of rejection there is gross villous shortening, the vascular lumens are entirely occluded and there is total destruction of the mucosa and muscular layers of the bowel wall with widespread fibrosis. The native gut, however, appears normal.

In the one-way GVHD (Lew $\rightarrow$ $LBNF_1$) model, the response has similarly been well described (144,151-153). It follows a predictable pattern which primarily involves the skin (dermatitis), the host lymphatic tissues (lymphocyte depletion and loss of follicular architecture) and the host bowel (necrotizing enterocolitis). Clinically in the rat, this onsets with redness and swelling of the ears, dermal erythema and scaling, alopecia and diarrhea, with marked hepatosplenomegaly, progressing to rapid weight loss and death by POD 14 . Skin biopsies from the dermal-epidermal junction reveal lymphocyte infiltration and basal cell degradation, without associated squamous epithelial changes (151). Histopathologically, the host bowel develops a severe enterocolitis, onsetting by POD 9 to 12. This is

manifested by a generalized mononuclear cell infiltrate of the LP followed by patchy crypt necrosis, clubbing of villi with sloughing of their tips and progression to a fulminant necrotizing enterocolitis by POD 14 (151,154). Grossly, it becomes dilated, thin and gaseous (154). The grafted bowel, however, appears spared. Changes in graft and host lymphoid contents and architecture are evident by POD 5 with progressive lymphoid depletion and loss of follicular architecture of the graft PP and MLN, and the host lymph nodes, spleen and thymic tissue (151). There is an initial proliferation of histiocytoid cells and immunoblasts seen in these lymphoid tissues. These subsequently disappear, over days, as lymphoid architecture is lost (144,151).

Donor T-cells are absolute necessities for GVHD to develop (149,155,156). Studies, outlined in more detail below, have shown that depletion of these immunocompetent graft cells prior to transplantation can significantly increase survival. Moreover, the alloreactivity of the transplanted T-lymphocytes is a strong determinant of whether GVHD will develop, as some cell subsets appear to be more alloreactive than others. For example, hepatic T-cells in orthotopic liver transplant models do not induce a lethal GVHD either *in vivo* or as manifested by *in vitro* mixed lymphocyte culture (MLC) studies, contrasting the reactions seen with SBT models (157). In addition, the rat strain combinations used can determine the presence or strength of a reaction (158).

The significance of GVHD in SBT has been questioned, however. In the two-way rejection models, GVHD responses are mild, limited and not a lethal problem as compared to HVG rejection (144,159). In large animal studies, however, the picture is less consistent, and some studies have reported variable intensities of GVHD responses, primarily in the canine model (160,161). However, such large animal models are not well-defined immunologically, and simultaneously occurring HVG rejection responses and GVHD interfere with each other and cannot truly be well characterized (153).

In the limited human experience so far, most recent reviews have not listed GVHD as a clinical problem. It has been reported in only one case of a series of 30 from Pittsburgh (a patient who died 23 days post-operatively and was also found to have significant rejection of her graft) (131) and in none of

the 9 patients transplanted in Paris (116). This may, however, be due to postoperative immunosuppressive regimens "holding GVHD responses at bay" and preventing the occurrence of any clinical manifestations.

Some question whether GVHD can even, pathophysiologically, occur. It can be argued that in an already enterectomized patient, host MLN and GALT is absent or markedly diminished; one has to wonder how "trafficking" of the corresponding donor lymphocytes can exert the negative effects expected in GVHD (162).

Indeed, the role of GVHD is still not well defined in clinical SBT. Some investigators feel that it is present in all cases of SBT but is masked by the intense and prolonged immunosuppressive regimens employed. Perhaps it need only be looked for (163). GVHD responses are immunosuppressive in themselves and some feel that some degree of GVHD may help to mitigate rejection and thus facilitate engraftment (152,164). With a further understanding of the role of GVHD in SBT, it is conceivable that such immunological responses may be used advantageously as part of immunosuppressive therapy. Unfortunately, however, GVHD-induced immunodeficiency states have also been found to correlate with a high susceptibility to infections and the development of malignancies. Combined with strong immunosuppressive therapy, such responses may contribute to the development of fatal sepsis and PTLD (165,166).

Immunosuppression and the Induction of Tolerance

One of the largest stumbling blocks to clinical SBT has been the development of adequate immunosuppressive regimens and the induction of host "tolerance" to the allografts, defined as the acquisition of nonreactivity towards donor-specific antigens (167). Considering the unique immunologic aspects of small bowel allografts, it would appear that there may be two general approaches to immunosuppression for SBT: to physically or functionally reduce the load of immunocompetent cells pre-transplantation, thereby eliminating the potential for GVHD, and to immunosuppress the lymphoid responses of the recipient to eliminate HVG rejection.

Reduction of Allograft Immunocompetent Cell Load: As discussed earlier, the large load of foreign immunocompetent cells carried by the allograft into the recipient has the potential for inducing GVHD. Various modes of graft pre-treatment to reduce or eliminate this load have been investigated. Experimentally, such methods can diminish GVH responses and reduce the requirements for long-term immunosuppressive regimens needed to curtail late GVHD.

Manual removal of the MLN has been shown to circumvent GVHD better than sublethal irradiation and administration of CsA in a one-way GVHD rat model (153). This was also demonstrated by Piernne *et al* who hypothesized that the GALT lymphocytes, which are still transplanted, play a less significant role than those in the MLN in the development of acute GVHD (154). Indeed, *in vitro* studies have shown that the MLN are the most potent stimulators and responders in one-way MLC as compared to other lymphocyte populations of the small intestine (168). However, these studies do not rule out the possibility that these other lymphoid compartments may play a role in the more chronic form of GVHD, in which the humoral component of the response may be more predominant. Also, in interpreting these studies, it must be noted that rats have a more developed MLN component compared to higher species, which may explain some of the discrepancies seen in large animal studies, and question the clinical applicability of this procedure.

Hematopoietic cells are radiosensitive. *In vitro* irradiation of the graft pre-transplant, in an effort to inactivate donor lymphocytes, has been investigated as a mode of controlling GVHD. Doses of 50 cGy in dogs (161) and 950 to 1000 cGy in one-way GVHD rat models (153,169) have been successful in averting GVHD. More recent studies by Hasuike *et al* have shown that GVHD can be successfully averted in unidirectional GVHD rat models with selective MLN irradiation, so as to spare the radiosensitive intestinal enterocytes from any radiation-induced damage (170). But, as discussed above, the roles of MLN lymphocytes versus those in the GALT (not irradiated in this study) in GVHD responses have not yet been fully elucidated.

Studies by Stangl *et al*, however, using both *in vitro* irradiation and *in vivo* whole-body irradiation of the donor with 1000 cGy, failed to control HVG rejection responses in a fully allogeneic rat SBT model, although still able to achieve profound lymphopenia in the intestinal graft (and the donor) (171). They feel that the small bowel in rats contains a large number of class II-positive, radio-resistant antigen-presenting cells (macrophages, dendritic cells) which play a major role in the rejection process and which are not defunctioned by the irradiation process. Furthermore, addition of CsA immunosuppression to pre-transplant graft irradiation has not been shown to significantly improve survival in fully allogeneic rat or pig models, and in fact, may have a negative impact on survival when used in high doses (172,173). Thus in fully allogeneic SBT, graft irradiation pre-transplant appears to have no benefit. In addition, the long-term nutritional consequences of intestinal irradiation (174) or possible disruption of the protective barrier function of the bowel wall may clinically limit this procedure.

Chemical depletion of the lymphoid tissue responsible for GVHD, more specifically the immunocompetent donor T-lymphocytes, has been experimentally more successful. Polyclonal antilymphocyte serum (ALS) acts in such a manner, by profoundly depleting tissue and peripherally circulating lymphocytes (175). Schaffer *et al* clearly demonstrated that pre-treatment of the donor with ALS prevented the occurrence of GVHD with no negative effects on weight gain and absorption of dietary nitrogen and energy in a one-way GVHD rat SBT model (175). When used in conjunction with CsA, neither GVHD nor HVG rejection were seen in a fully allogeneic combinations of SBT, and graft survival was significantly prolonged (159,176,177). Similar studies using pre-transplant treatment of the donor with R73, an anti-pan-T-cell monoclonal antibody, have revealed prolonged survival with curtailment of GVHD in a unidirectional GVHD rat model (178). Again, this is associated with marked depletion of T-cells in donor peripheral blood and mesenteric lymph nodes.

More recently, efforts have focused on the determination of the individual biological, functional and immunogenic properties of the various cell populations resident in the MLN and GALT. Indeed, these may have diverse roles to play in the immune interactions occurring in HVG and GVHD reactions.

In vitro studies have demonstrated the strong immunogenicity of the B-cell and dendritic cell populations in the small bowel allografts, as compared to weaker immune responses towards LP lymphocytes (179). It would appear therefore, that the process of intestinal rejection appears to be initially targeted to the PP and MLN (180,181). Thus, efforts to reduce the immunogenicity of the allografts should perhaps be directed towards these specific cell populations.

Over two decades ago, Ruiz and Lillehei suggested that, "when intestinal allotransplantation is used to maintain nutrition, in man, there would seem to be no need to use the entire small intestine of a cadaver; 1 or 2 meters should be sufficient" (121). Subsequently, studies have shown that use of segmental intestinal allografts confers a significant immunologic advantage towards reducing the risk of GVHD as well as host acceptance of the graft. Indeed, GVHD was not seen when Kimura *et al* transplanted 20 cm of proximal jejunum in a semiallogeneic GVHD SBT rat model (182). In a fully allogeneic model, similar allografts are rejected by the host, however, with the administration of low-dose CsA, mean survival time (MST) was significantly prolonged to 91 days (183). This length of bowel, which represents approximately 20% of the total intestinal length, has been shown to be able to adapt and allow for normal growth and development in rats, although extrinsic vitamin B12 administration is necessary to avoid fatal megaloblastic anemia (183,184). In models of extreme SBS, rats who also lack the ileocecal valve and colon may require longer lengths of bowel (40% of total length) to permit adequate nutrition and growth (185). Similar results have been obtained by others in rats (186,187) and in large animal models of dogs and pigs (188,189).

Segmental small bowel allografts offer several advantages besides the obvious immunological consequences. Acute rejection episodes are less frequent, and, if they occur, are less dramatic than with total SBT (188,189). Reduction of immunosuppression dosages can also be achieved with segmental allografts (183,190). More significantly, perhaps, is that application of this to clinical transplantation may allow for use of living HLA-related donors. Indeed, in canine studies, MHC-matched segmental

small bowel allografts underwent no rejection episodes with significantly improved survival compared to mismatched transplants (191).

Immunosuppression of the Recipient: Allograft pretreatment can diminish GVHD responses and possibly reduce immunosuppression required, but has little effect on the host rejection of the graft itself. Also, as discussed above, the role of GVHD in clinical SBT is still not well defined. Thus, most therapeutic efforts in SBT have been directed towards the treatment of the host to permit initial acceptance of the graft and induce long-term donor-specific tolerance. The mainstay of such treatments involves the administration of immunosuppressive agents including various drugs and monoclonal antibodies targeted to specific steps in the immune response (Figure II-1). Other modalities include pre-transplant donor-specific transfusions and co-transplantation of other "tolerogenic" organs such as the liver.

IMMUNOSUPPRESSIVE AGENTS FOR SBT

THE EARLY ERA OF IMMUNOSUPPRESSION

The early era of experimental and clinical SBT utilized conventional immunosuppressive regimens involving 6-mercaptupurine azathioprine, corticosteroids and antilymphocyte sera (ALS) (115,121,122,192). However, outcomes were poor because of consistent rejection of the allografts. Significant progresses in immunosuppression was obviously needed before SBT could become a realistic option for the treatment of SBS.

CYCLOSPORIN

With the discovery of Cyclosporin A (CsA), the second era of immunosuppression for SIT began. Indeed, it is with no doubt that the modern period of transplantation of all vascularized organs was marked by the discovery of this powerful agent which has since become the mainstay of a wide range of immunosuppressive regimens. Excellent reviews on the experimental development and uses of this drug have been published. (193-195).

CsA is a metabolic product of the fungus *Tolypocladium inflatum Gams*, and has been widely used for the treatment of transplant rejection and autoimmunity. It has been shown to block T-cell activation, primarily by preventing the production of lymphokines at the transcription level (196).

Mechanisms of Action

Innumerable studies have led to the elucidation of the molecular basis on which CsA exerts its immunosuppressive effects (196-201). It acts as a prodrug, which complexes to a 15-17 kDa intracellular binding-protein (or "immunophilin"), termed cyclophilin. This complex then binds to and blocks its phosphorylating function of calcineurin, an intracellular calmodulin-dependent protein phosphatase.

Early in the allograft rejection response, antigen presentation to the T-cell sets off a series of intracellular signals to activate calcineurin. Calcineurin can then phosphorylate the cytoplasmic subunit of a transcription factor for the interleukin-2 (IL-2) gene, NF-ATc, allowing it to translocate to the nucleus and combine with its corresponding nuclear subunit, NF-ATn. The resultant functional transcription factor, NF-AT, interacts with the Il-2 enhancer region, allowing for the gene's subsequent expression. Thus, by its inhibition of calcineurin, CsA inhibits Il-2 gene transcription upon activation of T-cells during a rejection, thereby inhibiting the early activation phase of the immune response.

Further studies by Zipfel *et al* (202) have elucidated that CsA may also inhibit the expression of over 10 genes induced *in vitro* after mitogen activation, including IL-2, IL-3, IL-4, GM-CSF and IFN-γ. They hypothesize that these multiple genes, all required in the immunoactivity of stimulated T-cells, may share common activation components required for induction and therefore be commonly inhibited by CsA. In addition, CsA appears to decrease the responsiveness of T cells to IL-2 stimulation by both inhibition of IL-2-receptor expression and other less well characterized mechanisms (203).

Animal Studies in SBT

It was a landmark study by Reznik *et al* which demonstrated prolongation of small bowel allografts in the dog using CsA (117). Using a dosage of 25 mg/kg/d, intramuscular (i.m.) for 28 days, then orally (p.o.), they were able to obtain a mean survival time (MST) of dogs given a total orthotopic SBT of 91

days compared to 12.5 days seen in control animals. However, of the three animals who survived long term (>200 days), two eventually succumbed to graft rejection, felt to be secondary to falling CsA levels. Of the eight dogs which died early (9 to 60 days post-transplant), none had any evidence of allograft rejection. However, five developed fatal pneumonia, reflecting the increased susceptibility to infection caused by immunosuppression. Further studies by this same group emphasize the importance of early parenteral administration of CsA; superior MSTs are obtained with early i.m. administration as compared to oral (204). This has been confirmed by others and is felt to be due to the dependence of CsA on bile solubilization as well as a markedly erratic absorption in cases of intestinal dysfunction (205-207).

Since then, the rat model has become the prototypical animal model for SBT because of the availability of inbred strains with which to study rejection responses. In rats, CsA can prolong survival of completely allogeneic small bowel transplants with a wide array of doses including 20 mg/kg/d, indefinitely, 15 mg/kg/d for 2 weeks, 15 mg/kg/d for 1 week followed by every alternate day for 4 to 5 weeks, and 5 mg/kg/d for only 2 weeks (208-211).

Short-courses of CsA appear to be able to induce tolerance, with long-term survival of the allografts after discontinuation of the immunosuppression. This has been observed in rats and dogs after 28 days and 3 months of treatment, respectively (207,209). It is felt that tolerance may develop in a similar manner as observed in liver transplant recipients (212). By a dose-dependent inhibition of T-cell lineages, CsA may suppress clonal expansion of (or may effectually eliminate) cytotoxic-T cells with a lesser effect on T-suppressor cells, thereby inducing a long-lasting unresponsiveness after withdrawal of CsA treatment. Indeed, such long-term effects of CsA on T-cell proliferation have been demonstrated *in vitro* (213). Other investigators have hypothesized, however, that an unstable tolerance mechanism, dependent on continuous exposure to alloantigen, or a serum factor mediating tolerance plays a role in the development of tolerance (214).

Clinical Experiences with Cyclosporin in SBT

Until the advent of CsA, clinical experiences with SBT had been universally unsuccessful. With its good results in transplantation of other vascularized organs, however, it was an obvious new agent to be trialed in clinical SBT.

The first reported case of SBT utilizing CsA was by Cohen *et al* in Toronto (215,216). Their case involved a 26 year old, blood group A woman with SBS who received the entire small bowel from a 10 year old, blood group O male donor. Immunosuppression consisted of CsA, 2 mg/kg, and solumedrol (30 mg) pre-operatively followed by CsA, 6 to 9 mg/kg/d in divided doses for 3 days then a continuous intravenous (i.v.) infusion of 4 mg/kg/d, and a tapering dose of solumedrol (to 10 mg, twice daily by POD 6). Unfortunately, however, the patient died rapidly by POD 11, with the graft revealing ongoing rejection and the possible development of CsA-induced hepatotoxicity and neurotoxicity.

The first successful isolated SBT under CsA was performed in Paris, France (124). This was a 5 month female infant who underwent a total small bowel and cecal resection at birth, and received an intestinal allograft from an anencephalic neonate in March of 1989. Immunosuppression consisted of pre- and post- operative CsA and solumedrol with anti-thymocyte globulin (ATG) and azathioprine added post-operatively. Despite 3 rejection episodes, all successfully treated with OKT3, anti-IL-2-monoclonal antibodies and ATG respectively, graft survival has persisted with reportedly normal digestive function. At last report, 4 1/2 years later, the patient is growing and developing normally while being maintained on oral CsA and low-dose azathioprine and methylprednisolone (125). It must be noted, however, that part of the success of this transplant is likely due to the fact that the allograft came from an anencephalic neonate. Being relatively poor in $CD3^+$ lymphocytes, and having a shorter length with subsequently less lymphoid tissue, such an allograft is less immunocompetent, and specifically, markedly less able to mount a GVHD response. In addition, the short duration of TPN required prior to transplantation resulted in a relatively healthy recipient, with few TPN-related problems, hightlighting the importance of early surgical management in patients with SBS. Finally, the long-term CsA in this child has not induced any renal

dysfunction, although it has caused extensive hypertrichosis and severe gingival hypertrophy, contributing to eating problems; she subsequently requires nocturnal supplementation of an otherwise totally enteral diet. CsA peak whole blood levels are maintained at 600-700 μg/L, and trough levels at 100-150 ug/L, by radioimmunoassay (RIA).

The first successful SB/L transplant was also performed under CsA immunosuppression (126). Since then other SB/L as well as MV, or cluster, transplants have been managed with CsA, however, unwanted adverse effects of immunosuppression such as the development of infections and malignancies are not uncommon. To date, Grant *et al* report two successful SB/L transplants (well at 5 and 4 years) using CsA (127). However, in this series, there was also one sole small bowel graft which was rejected at 14 days and removed, one SB/L patient who died of CMV sepsis after 3 months, and two cases of cluster graft transplants who succumbed to lymphoma at 9 and 7 months respectively under this agent. Foster *et al* report similar results with two cases of MV transplantation under CsA: one patient eventually succumbing to lymphoma and the other doing well 5 months post-transplantation (136).

There have been nine attempted SBTs under CsA immunosuppression by Revillon *et al* in Paris (116). Poor results, however, with only one long-term survivor, have led the investigaotors to conclude that much improvement in immunosuppression is required before continuation of their SBT program (217).

It must be noted that, overall, the number of cases employing CsA have been small, and few of these have been done recently enough to enjoy the advances in other aspects of SBT that newer agents have (technical, rejection monitoring, etc.). Thus, comparison of the efficacy of CsA to other agents in clinical SBT cannot be appropriately made without controlled clinical trials.

Adverse Effects of Cyclosporin

Nephrotoxicity is generally considered to be the most significant toxic effect of CsA limiting its use. This is a dose-related effect, and usually responds to a decrease in CsA dose. In both rodents and humans, this appears to be caused by a dose-dependent increase in renal vascular resistance, a decrease in renal

blood flow and a decrease in glomerular filtration rate (GFR) (218-221). It is hypothesized to be due to CsA-induced injury of renal endothelial cells, resulting in an increase in thromboxane-A_2 and prostaglandin I_2 release. As well,CsA may induce vasoconstriction by increasing endothelin synthesis and cytosolic calcium, such that the contractile response of the renal vascular small muscle and mesangial cells to vasoconstrictor hormones is enhanced (220,222). These changes are reversible upon withdrawal of the drug; however, irreversible tubular structural and functional changes, possibly related to increased intracellular protein degradation, can occur with chronic administration at high doses (218,223,224). In addition, CsA's toxic effects on the renal tubules may lead to magnesium wasting, subsequently contributing to its neurotoxic effects (225).

These vasoconstrictive effects are widespread and may lead to accelerated hypertension. One study demonstrated clinically significant hypertension onsetting within 1 week post-transplantation and reaching levels greater than 140/90 mmHg within 4 months, in 78% of patients immunosuppressed with CsA (221). This was associated with an increase in systemic vascular resistance (SVR) and concomitant decrease in cardiac index (CI).

Numerous other unwanted effects of CsA therapy have been observed (195,226,227). Neurotoxicity is frequently reported, and can occur in up to 20% to 36% of renal and liver transplant patients (228,229). However, this usually develops in only the first three months of therapy, primarily involves only mild tremulouness, and only rarely (1 to 2%) manifests as severe symptoms of paresthesias, depression, somolence or seizures (229). Cerebral pathology can be seen on computed tomography with the appearance of white-matter hypodensities, suggesting increased water content in the brain (195).

Hirsuitism and gingival hyperplasia are also common, and can occur in up to 30% to 50% of patients (125,229,230). The latter can be particularly troublesome in SBT as it may be associated with eating difficulties as well as undesired cosmetic appearances (125). Gynecomastia can also occur.

CsA can have a profound effect on carbohydrate metabolism, with a 40% incidence of impaired glucose tolerance induced in liver transplant patients (231). It is moderately diabetogenic secondary to an

inhibition of insulin mRNA production and subsequent release of insulin from islet cells (195,232). It may also increase peripheral insulin resistance, resulting in persistent hyperglycemia (233,234). Other metabolic effects include increases in serum cholesterol and uric acid levels (227).

A high risk of infectious complications is a unfortunate reality of any immunosuppressed state. This seems especially so in the SBT patient who is at risk for indwelling catheter (for TPN) infections, bacterial translocation from a rejecting graft, and CMV enteritis. Because of immunosuppression, such cases may develop into fatal sepsis. Immunosuppression also leaves the patient at risk for the development of malignancies. Indeed, as discussed above, the development of PTLD is not uncommon in SBT patients treated with CsA (127,136).

Gastrointestinal effects, including diarrhea, nausea, vomiting and abdominal discomfort, are also common. However, they are usually attributed to the oil-based vehicle used in oral preparations, and can be ameliorated by use of gelatin capsules (195).

Dose-related hepatotoxicity is seen as manifested by elevated transaminases and γ-glutamyltransferase levels. The development of cholestasis with a subsequent reduction in fat absorption is common (235-237). This adverse effect on bile secretion does not appear to be due to direct effects on liver function enzymes, nor light or electron microscopic hepatic architecture (235,236,238). Rather, it appears to be secondary to CsA's inhibitory effect on the ATP-dependent bile salt transporter in the liver canalicular membrane (239-241). Nutritionally, this could affect the emulsification and subsequent absorption of dietary fats.

Finally, allograft acceptance is not the only desired outcome of SBT; a **functioning** graft is essential. The transplant process itself, by virtue of denervation, disruption of lymphatics, and other less well defined mechanisms, can have a profound effect on allograft function (242-250). It is therefore necessary that the immunosuppressive regimens utilized for SBT have no or as little effect on bowel function as possible. However, in addition to the hepatotoxic and cholestatic effects of CsA, this agent has been shown to adversely alter some aspects of small bowel physiology. Sigalet *et al* have shown that moderate

doses of 15 mg/kg on alternate days reduces *in vitro* active uptake of glucose and passive uptake of long-chain fatty acids (linoleic and stearic) in rats (251). At double this dose, similar *in vitro* effects are seen, as well as a reduction in weight gain and *in vivo* nutrient absorption (212,251,252). Earlier studies by Collin *et al* have also shown a reversible reduction of *in vivo* glucose, alanine and lauric acid absorption in autotransplanted dogs given CsA (253). These effects may be partially due to CsA's effect on the Na^+-K^+-ATPase glucose cotransporter at the enterocyte level, as seen with renal tubular cells (227), which is the main mechanism of glucose uptake in the small intestine (255,256).

CsA has also been observed to alter intestinal permeability. In normal rats it can induce an increase *in vivo* permeability to [^{51}Cr]-EDTA, indicating an increased permeability of intracellular tight junctions (257). This may reflect a change in the composition of the enterocyte membrane by CsA which would in turn alter nutrient uptake (258).

Changes in bowel morphology have been reported with this agent. Heeckt *et al* observed smooth muscle hyperplasia, with up to 2-fold increases in cell numbers in the longitudinal and circular muscle layers in normal rats treated with long-term CsA (259). However, on a functional level, carbachol-stimulated contractile activity was not altered by this proliferation. Sigalet *et al* also observed an increase in jejunal villus width and ileal villus height and width in rats treated with high dose CsA (252).

THE NEW ERA OF IMMUNOSUPPRESSION

The advent of CsA was certainly a milestone in the transplant of all vascularized organs, including the small intestine. However, as already discussed, the strong immunogenicity of the small bowel may demand more specific and powerful immunosuppression regimens, without a concomitant increase in toxicity. Hence, over the past decade, there has been the development of a variety of new immunosuppressive agents with distinct and diverse modes of action in controlling allograft rejection. By understanding not only the immunological and clinical effects of these drugs, but also their cellular and

molecular mechanisms of action, there is the potential to use them in combination to act at varying points in the immunological processes of allograft rejection.

FK506

FK506 (Tacrolimus) is a macrolide antibiotic, first discovered in 1984 when isolated from cultures of *Streptomyces tsukubaensis* (260). It is a newer agent, presently used as an immunosuppressive agent in experimental and clinical transplantation. *In vitro*, FK506, like CsA, inhibits IL-2 production and IL-2 mRNA synthesis, however, it appears to be almost 100 times more potent than CsA (261,262). *In vivo*, its potency to suppress cell-mediated and humoral responses is at least 10-fold greater than that of CsA (262).

Mechanisms of Action

FK506 has a different molecular structure from Cyclosporin, but appears to act by the same mechanism (196-199). By binding to its corresponding intracellular immunophilin, FK-binding-protein (FKBP), it also inhibits the phosphorylating activity of calcineurin, and subsequently, inhibits the transcription of the IL-2 gene in immune-activated T-cells. It also inhibits the synthesis of mRNAs of other genes up-regulated during T-cell activation, including IL-3, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-stimulating factor (GSF), tumor-necrosis factor-α (TNF-α), and interferon (IFN)-γ and -α (263). In addition, FK506 decreases the responsiveness of T-cells to IL-2 stimulation by both inhibition of IL-2-receptor expression and other less well characterized mechanisms (203). Thus, like CsA, it acts in the activation phase of the immune response.

FK506 may also have some immunoregulatory actions distinct from CsA (264). It appears to have an inhibitory effect on B-cell function. Studies have shown that it inhibits cell proliferation of B-cells by selectively blocking the G_0- to G_1-phase of the cell cycle. B-cells progressing from the G_1 to S-phase are also sensitive to the inhibitory action of FK506. These actions are different from that of CsA which does not suppress the proliferation of activated human B-lymphocytes (265).

Animal Studies in SBT

With its success in inhibiting rejection of renal, cardiac and liver allografts (266-268), FK506 has been used in animal studies of SIT. Hoffman *et al* from Pittsburgh were one of the first to demonstrate the ability of FK506 to prolong survival of small intestinal allografts in rats (269). Using a dose of 2 mg/kg/d for 6 days then every alternate day for 24 days, a MST of 51 days was achieved. However, 50% of the rats eventually succumbed to chronic rejection. No acute rejection episodes, GVHD or toxic effects of FK506 were noted. Further studies by this group using the same dose, but for only 4 days, showed long-term (greater than 180 days) functional graft survival in 5 of 5 fully allogeneic transplanted rats with no signs of rejection or GVHD; one rat eventually died from pneumonia, however (270).

With experience, the toxicities of FK506 were becoming realized, and lower dosage schedules developed. Hatazawa *et al* were able to obtain long-term survival in fully allogeneic transplanted rats with doses of 1 mg/kg/d for 8 weeks (271). No signs of rejection or GVHD were noted. A much lower dosage schedule, developed by the Pittsburgh group, 0.32 mg/kg/d for 13 days, significantly prolonged allograft survival (greater than 175 days) in 80% of rats transplanted across a minor histoincompatibility combinations and allowed for induction of donor-specific unresponsiveness (DSU) as tested by skin grafting after SBT (272). Across a major histoincompatible combination, however, allograft rejection could only be delayed, but not prevented, and DSU could not be induced. Others have had similar results with doses ranging from 0.1 to 0.5 mg/kg/d (273). In a one-way GVHD model (Lew $\rightarrow$ $LBNF_1$) GVHD is not seen with these low doses, clinically or histologically (274).

Clinical Experiences with FK506 in Transplantation of the Small Bowel and Other Organs

FK506 has been shown to be clinically successful in rescue therapy for failing liver grafts not responding to conventional immunosuppressive therapy of CsA, OKT3, steroids and azathioprine (275-279). Complete or partial success rates have ranged from 50-81% (275,277). The better results occur in patients with refractory acute rejection or "early" chronic rejection as opposed to long-standing chronic rejection. Similar results have been published on a large series of patients by the US Multicenter FK506

Liver Study Group (280). Randomized trials comparing FK506 to CsA as primary therapy for liver transplantation have been done. A large amount of this work has been published from Pittsburgh, who found a significantly prolonged graft survival and rejection-free period with FK506 (281). A series from Dallas, however, could find no significant differences between the two treatments (282).

Despite the significant nephrotoxicity of FK506, it has been successfully utilized in renal transplantation as both primary immunosuppressive therapy, and shown to be as efficacious as CsA (283-285) and as "rescue therapy" for ongoing acute rejection while on CsA immunosuppresion (286). As primary therapy, it has been found to be equally effective as CsA in a controlled clinical trial, with no significant differences in renal toxicity (285). FK506 has also been efficacious in preventing rejection of other transplanted organs, including heart, lung, and pancreatic islets (227,287,288).

In pediatric transplantation of liver, kidney, heart, lung, and liver-islets, FK506 has been reported as relatively "safe", despite a 8% incidence of post-transplantation lymphoproliferative disorder (PTLD) in one series (287). Others, however, have reported 74% and 22% incidences of major adverse effects and PTLD, respectively, when FK506 is used as "rescue therapy" for uncontrollable acute rejection of liver allografts under CsA primary immunosuppression (289). The most recent published series, by Rosh *et al*, of pediatric patients recieving FK506 for primary and rescue therapy for liver transplantation, reported a 17% incidence of PTLD (3 of 17 children); one out of these three cases was fatal (290).

With these reported clinical results and the success of FK506 in animal models of SBT, it has been used by several centers for immunosuppression of SBT alone, in combination with the liver, or as part of a multivisceral graft. The Pittsburgh group has published the largest series, the most recent being a 3 1/2 year accumulation of 61 cases (132,133). Immunosuppression was with FK506 (0.1 to 0.15 mg/kg/d, i.v. or 0.3 mg/kg/d, p.o.) plus steroids, prostaglandin-E1 and occasionally azathioprine, with rejection episodes treated by increasing the FK506 dose, steroid boluses or OKT3 (132). As described in an earlier section, 22 patients received an isolated intestinal graft, 26 received a combined small bowel-liver graft and 11 received a multivisceral organ graft, with a minimum follow-up of 2 months. One year graft

survival rates were 70%, 73% and 80% for the SB, SB/L and MV grafts, respectively. However, overall, the 24 month graft survival has been reported as only 53%. Fatal complications included acute rejection with sepsis, frequently worsened by CMV infection, sepsis secondary to technical failures or TPN catheter infections, pneumonia and the development of PTLD. These resulted in 5 deaths in the SB group (23%), 10 in the SB/L group (38%) and 2 in the MV group (18%). Functionally, 34 patients are TPN-free and 4 are on partial TPN only.

Grant *et al* reports two recent SB/L transplant patients (one child and one adult) treated with FK506 immunosuppression. Both are doing well at 6 months, however, follow-up is relatively short up to this time (127).

Thus, it would appear that FK506 can be successfully used in clinical SBT. However, caution must be maintained, as there is still relatively few long-term follow-ups and no real comparison can be drawn with other more conventional agents such as CsA, as comparable large series have not been reported.

Adverse Effects

The toxicity profile of FK506 has been well studied. Because of its similar mechanism of action as CsA, it appears to qualitatively have similar adverse effects.

Like CsA, FK506 can induce reversible nephrotoxicity (284,291-293). Several studies have been recently published, comparing the nephrotoxic effects of the two agents. Just as significantly as CsA, FK506 has been found to induce marked renal vasoconstriction, resulting in a significant decrease in GFR in liver transplant patients (221). The mechanism by which this occurs is controversial, however. Benigni *et al* have shown *in vitro* that unlike CsA, FK506 does not induce endothelial release of vasoactive mediators (TxA, PGI and endothelin) and thus may have a different mechanism by which it exerts its effects (220). This is difficult to interpret, though, as this same group did not observe the *in vivo* renal vascular hypertension, decrease renal blood flow and decreased GFR seen in human studies. Conversely, Moutabarrik *et al*, and others, have shown that both agents have a stimulatory effect *in vitro*

on endothelin secretion and nephrotoxicity is correlated with an increased production of thromboxane A_2 (260,294).

McDiarmid *et al* found no significant differences in renal function between CsA and FK506-treated liver transplantation patients as assessed by serial measurements of GFR up to 360 days post-transplantation (295). However, several studies have shown that clinically significant systemic hypertension is seen less frequently with FK506 immunosuppression than CsA (221,227,291). McDiarmid also noted such a trend, but the difference between the two treatments and the need for antihypertensive medication was not significant (295). Finally, as with CsA, hyperkalemia secondary to a type IV renal tubular acidosis can also occur with FK506 (291).

Vasculitis in the heart of renal-transplanted dogs receiving FK506 has been observed, and is of some concern (296). This appears to be most prominent when the dogs are given subtherapeutic doses of drug, and can be suppressed by co-administration of CsA, thereby, improving immunosuppression. Thus, the vascular toxicity induced by the agent may be potentiated by systemic immunological events of rejection. Two large Japanese studies noted a significant incidence of cardiac symptoms in renal transplant patients on FK506 (284). In the earlier study, with less stringent controls on serum levels, cardiac symptoms developed in 27.8% of patients, including chest pain in 13.9% and an abnormal ECG in 5.6%. In the later study, with whole blood trough levels maintained at 15-20 ng/mL, cardiac symptoms developed in 18.6% of patients, with 10% and 5.7% experiencing chest pain or abnormal ECG changes, respectively. These findings may have negative implications on the use of FK506 in patients with pre-existing ischemic heart disease.

The spectrum of neurotoxicity seen with FK506 also seems qualitatively similar to CsA (227,291). Manifestations range from minor tremors, headaches, sleep disturbances, dysesthesias and mood changes to major akinetic mutism, seizures, psychosis, encephalopathy, focal deficits and movement disorders (292,297). Eidelman *et al* found a frequency of 20% of minor disturbances and 5.4% of major manifestations in a large series of transplant recipients on FK506, most of whom had received either a

liver or cardiac allograft (292). These were more frequent during the i.v. phase of drug administration, early on in the immunosuppressive regimen, and commonly associated with drug trough levels significantly above the normal therapeutic level (293). More recently, Backman *et al* reported a 68% incidence of neurotoxicity in liver transplant recipients, with 35 of the 40 patients developing only moderate to mild symptoms, not related to elevated plama levels of the agent (298). This is a much higher incidence than that reported with CsA (228,229). Symptoms are dose-dependent, and usually respond to a reduction or temporary withdrawal of the drug. Pathologically, imaging studies show abnormalities of the cerebral white matter, central pontine myelinolysis or hemorrhage, similar to those seen with CsA neurotoxicity (292,299).

New onset diabetes mellitus has been reported with FK506 immunosuppression similar to that seen with CsA. Tabasco-Minguillan *et al* reported long-term insulin requirements in 39 patients receiving 52 orthotopic liver transplants in Pittsburgh (300). New onset diabetes requiring insulin administration occurred in 9 patients (23%) by 3 months post transplant, and diminished to 5 patients (13%) by 2 years. Another series of liver transplant recipients studied by the Pittsburgh group revealed a 36% overall incidence of insulin requirements (151 of 370 patients) which decreased to 12.1% requiring insulin on a long-term basis (297). An earlier series compared the diabetogenic effects of FK506 with CsA in a randomized clinical trial of primary liver transplantation and showed that the incidence of new-onset diabetes mellitus was similar in both groups, approximately 27% (281). More recently, others have shown the incidence of glucose intolerance and diabetes to be twice as great in FK506-treated liver transplant patients compared to those receiving CsA (231). This diabetogenic effect of FK506 has been studied by Ueki *et al* in a rat model (301). They have demonstrated ultrastructural changes and degranulation in islet beta-cells, suggesting that FK506 may interfere with protein (insulin) synthesis.

Other metabolic side effects of FK506 include hyperkalemia secondary to renal dysfunction as well as the development of hypercalcemia (291) and hyperuricemia (284). The incidence of hypercholesterolemia

and hyperlipidemia is varied. It has been reported as minor and less frequent than seen with CsA by some investigators (227,284,302) and the same as seen with CsA by others (291).

As with CsA, malignant and infectious complications can result from immunosuppression by FK506. However, data may be difficult to truly interpret as most patients receive immunosuppressive protocols involving other agents which may contribute to these complications. Fung *et al* examined a large series of patients from Pittsburgh on FK506 for both primary and rescue therapy (297). A total of 16 patients out of 1057 (1.5%) developed PTLD, 5 being fatal. The other nine were relatively mild forms treated with a reduction in immunosuppression with or with out acyclovir, or with surgical resection. However, as discussed above, children may be more susceptible to the development of PTLD under FK506, with an incidence of 8% to 22% reported in some series (287,289).

CMV infections appear to be the most frequently seen opportunistic infections among transplanted patients . In this same series, the incidence of CMV infections in FK506-treated patients was about 20%, and is felt by the authors to be similar to that seen in transplanted patients on CsA (297).

Other less significant adverse effects include gastrointestinal symptoms ranging from minor cramps and abdominal distention to nausea, vomiting and diarrhea (284). In some patients, however, these may evolve into major diarrhea, malabsorption and malnutrition problems, not resolving to lowering of drug dosages, and necessitating conversion to another immunosuppression regimen such as CsA. This has been recently reported by Mor *et al* in three liver transplant patients with 'desirable' FK506 plasma levels of 0.5 to less than 5.0 ng/mL (303). Finally, hot flushes, pruritis and alopecia are also seen in a few patients (297).

RAPAMYCIN

Rapamycin (RAPA) is a macrolide lactone, similar in structure to FK506. It was first isolated over 15 years ago as an antifungal antibiotic from *Streptomyces hygroscopius*, a soil bacterium from Rapa-Nui, Easter Island, and was subsequently found to have immunosuppressive properties (199,304,305).

Mechanisms of Action

Although it is structurally related to and binds to the same intracellular receptor as FK506 (FKBP), RAPA acts by a different mechanism than either FK506 or CsA (199,306-310). However, like these agents, its immunosuppressive actions are T-lymphocyte-specific. CsA and FK506 exert their function by inhibition of the production of IL-2 (and a limited set of other lymphokines) required for T-cell proliferation and differentiation, thus inhibiting early events of T-cell activation. RAPA, however, does not interfere with IL-2 production nor IL-2 receptor expression, but rather, blocks the proliferative response of activated T-cells to IL-2 and other cytokines (311).

Although a direct target for the action of the rapamycin-FKBP-complex has not been defined, it appears to inhibit some biochemical event(s) necessary for the progression of IL-2-stimulated T-cells from G_1- to S-phase (306,307). *In vitro* studies by Morice *et al* have shown that RAPA inhibits growth of an IL-2-dependent cytotoxic T-cell line in the late G_1-phase, just prior to entry of the cells into the S-phase (312). It is postulated that this inhibition is related to suppression of the late G_1-phase increase in $p34^{cdc2}$ activity, normally induced by cellular stimulation with IL-2. $P34^{cdc2}$ is the prototype cyclin-dependent kinase involved in highly conserved regulatory cascades which control both the G_1- to S-phase and G_2- to M-phase transitions in cell proliferation. Inhibition of the activity of this kinase has been demonstrated in both fibroblast (312) and myogenic cell lines (313).

The exact nature of how RAPA inhibits the activation of $P34^{cdc2}$ is not yet understood. Studies indicate that RAPA may target upstream events required for this process (312). Recently, RAPA has been shown to inhibit IL-2 induced activation of the p70 S6 kinase in both T-cells, mast cells and fibroblast cell lines (314-316). P70 S6 kinase may be involved in the transduction pathway leading to $p34^{cdc2}$ activation and S-phase commitment in T-cells (312). Alternatively, p70 S6 kinase may be involved in an alternate signalling pathway in the regulation of T-cells into S phase. Activation of both kinases could therefore result from separate pathways initiated by a common RAPA-inhibitable molecular event (312). Although not yet fully understood, it appears that the immunosuppresive action of RAPA somehow relates to its

ability to inhibit late G1/S phase transition of IL-2 activated T-cells. Interestingly, it may also be involved in blocking signal transduction systems in non-lymphoid cells, such as myogenic and fibroblast cell lines as discussed above.

By virtue of their interactions with a common receptor site(s) on FKBP, RAPA and FK506 act as reciprocal antagonists of IL-2 secretion and T-cell proliferation *in vitro* (306,308). In contrast, RAPA acts synergistically with CsA *in vitro* to inhibit human peripheral blood lymphocyte function, including proliferation, generation of allo-cytotoxic-T-lymphocytes in mixed lymphocyte culture (MLC) and cytokine-driven proliferation of IL-6 or IL-2 dependent cell lines (317).

Animal Studies in Transplantation of the Small Bowel and Other Organs

The success of RAPA in prolonging rat heart and bovine kidney allografts (318) has led investigators to examine its effects in SBT, with a hope to develop much needed improved immunosuppressive protocols for intestinal transplantation. Stepkowski *et al* were among the first to demonstrate RAPA's ability to prolong survival of small bowel allograft recipients in a rat model (319,320). Using an i.v. dose of 0.8 mg/kg/d for 14 days, MST was extended to 26.8 days compared to 10.0 days in untreated controls. Further studies by this group used similar dosages to evaluate RAPA's effects in one-way rejection and GVHD models in the rat (321). In the GVHD model, graft survival was prolonged to 21 days compared to 12 days in untreated animals. In the HVG rejection model, allograft rejection was prolonged to 41 days with RAPA compared to 12 days in controls. The authors thus propose that RAPA protects small bowel allografts more effectively against HVG than GVH immune responses. Others have demonstrated similar results in semiallogenic models of SBT (322).

The *in vitro* synergism between RAPA and CsA, discussed above, has been demonstrated *in vivo* in a rat SBT model (323). Using low doses of both agents (RAPA, 0.2 mg/kg/d, i.v. and CsA, 2 mg/kg/d, s.c. for 2 days, then p.o.), the MST of fully allogeneic allografts was prolonged to 37 days as compared to 15 days and 16 days , respectively, when these drugs were used alone. Similar synergism has been demonstrated in rat cardiac transplantation models (319,324).

The use of RAPA in human trials has not yet been published, however, some large animal models have been developed with other vascularized organ transplants. Calne *et al* (318) were among the first to publish their results of significantly improved survival of pigs who received renal allografts under RAPA immunosuppression (2 mg/kg/d, i.m. for 64 days). Three of nine animals survived over 8 months, with normal renal function. Five died after 7 to 9 weeks from interstitial pneumonitis, likely the result of over-immunosuppression. Similar results have been recently published by Almond *et al*, demonstrating prolonged survival of renal-transplanted pigs with 0.25 mg/kg/d, i.m., with a major cause of mortality being pulmonary infection (325).

Clinical Experiences with Rapamycin

Clinical trials with this agent are presently underway, but have not yet been published (326).

Adverse Effects

The toxicity profile of RAPA has not been as well established as CsA or FK506. It is not surprising that, considering its similar intracellular protein-binding ability as FK506, it also shares many common adverse features.

Dogs fare poorly on RAPA, developing vomiting and diarrhea, with pathological ulcers throughout the entire gastrointestinal tract secondary to an acute necrotizing fibrinoid vasculitis of the arterioles and small arteries, after daily oral doses of 0.25-5 mg/kg (318,327). Similar lesions develop even after a limited course of 2 mg/kg/d on days 3-5 (318). Interestingly, vasculitis has also been shown to be a major side effect of FK506 when used in dog renal transplantation (296). Furthermore, the G.I. vasculitis induced by RAPA may actually be quite mild (as seen in nontransplanted dogs) and may be severely aggravated by systemic immunological events of rejection (327). Correspondingly, synergistic, and thus, more immunosuppressive, combinations of low-dose RAPA with CsA reduced the frequency and severity of vasculitis as compared to low-dose RAPA alone (327). Such vasculitic lesion are not seen in the porcine model (325). It may be that this effect is a species-specific reaction. However, preliminary

studies in primates demonstrate that, although RAPA is immunosuppressive in renal transplantation models, severe GI vasculitis may develop, even at subtherapeutic doses (328).

These effects can lead to emaciation and death in dogs. In rats, although vasculitic GI ulcers have not been reported, RAPA also has negative effects on weight gain (329,330).

The renal effects of RAPA have been studied in rats by DiJoseph *et al* (268). They found that at high doses, significant renal dysfunction can develop in nontransplanted rats, although this appears to be strain-specific. Histologically, mild to moderate necrotizing vasculitis and tubular atrophy is observed. Whiting *et al* also noted a significant increase (albeit, minor) in serum creatinine in rats treated with 1.5 mg/kg/d, i.p., for 14 days (330). More significantly, however, they observe a further dramatic increase with a marked decrease in GFR when RAPA was administered in combination with CsA. They propose that RAPA may potentiate the well established nephrotoxicity of CsA when these agents are used in combination. In renal transplanted pigs given low but therapeutic doses (0.25 mg/kg/d, i.m. for 30 days), serum creatinine levels do not increase (325).

RAPA may also be diabetogenic. Whiting demonstrated that in rats, RAPA causes significant increases in plasma and urine glucose levels (330). Moreover, as with its nephrotoxicity, the addition of CsA, also known to be diabetogenic, potentiates this effect. They observed no histological abnormalities in the pancreases of the treated rats, however, hypothesize that this may be due to the administration of drug dosages below the level required to induce structural damage to the islet cells. These same investigators also noted the development of focal myocardial necrosis in rats treated with RAPA; and again, this effect was potentiated by CsA (but, not observed to occur with CsA alone) (330).

Other adverse effects of RAPA include an increased risk for bacterial infections (318,325,328). In large animals, significant thrombocytopenia may develop, even at low doses (318,325). Finally, unlike CsA and FK506, which have been shown to be hepatotrophic (227,331), RAPA significantly inhibits hepatic regeneration in rats in a dose-dependent manner. This was shown by Francavilla *et al* in rats who had been treated with RAPA; where DNA synthesis in the organ, post-partial resection, was significantly

inhibited relative to untreated controls (332). In a similar manner, RAPA also inhibits kidney and intestinal regeneration at doses as low as 0.3 mg/kg/d, i.v., for four days (332). Consideration of these negative effects in SBT may be warranted, especially if limited segmental allografts are considered.

15-DEOXYSPERGUALIN

15-Deoxyspergualin (DSG) is an antitumor agent isolated from the fermentation broth of *Bacillus laterosporus*, a soil bacterium isolated from a mountainous area in Japan, which has subsequently been found to have immunosuppressive properties (333,334). Its structure, a straight polypeptide guanidine derivative, is quite different from those of the previous immunosuppressives discussed: CsA, FK506 and RAPA are cyclic polypeptides. Likewise, its immunosuppressive effects are also very different.

Mechanisms of Action

In vivo studies in rats by Suzuki *et al* have suggested that DSG selectively inhibits activated donor-specific expanded lymphocyte clones at the onset of rejection (335,336). They also demonstrated that DSG may have no effect on suppresser cells, thereby allowing them to progressively develop and maintain a long-term state of immunologic unresponsiveness (335).

In vitro studies by Jiang *et al* showed that unlike CsA and FK506, which inhibit the early phase of the mixed lymphocytic reaction (MLR) (by inhibiting T-cell activation at the IL-2 production level), DSG acts later, primarily by inhibiting the expression of IL-2 receptors. It also suppresses cytotoxic T-cell generation (337). Similarly, Nichimurak *et al* demonstrated that DSG was able to suppress differentiation and proliferation of alloreactive cytotoxic T-cells, but unlike CsA, did not inhibit IL-2 production by T-helper cells (338).

Other effects of DSG include antimonocytic activity, with decreased expression of MHC class II antigens, IL-1 production, free radical generation and secretion of hydrolytic lysosomal enzymes by mouse peritoneal macrophages and human monocytes (339). DSG may also impair humoral immunity, making it a favorable agent to be used in xenografting (291,340).

Unfortunately, however, unlike the CsA, FK506 and RAPA, no definitive molecular mechanisms of action of DSG have been elucidated. It has been found to interact with a member of the Hsp70 family of heat shock proteins, which may represent a new class of immunophilins (341). Like the immunophilin isomerases cyclophilin and FKBP, heat shock proteins can act as "foldases", which may represent a common mechnism of action of these immunosuppressive agents. Alternatively, like isomerases, they may act as carrier molecules to translocate DSG to the nucleus or other organelles where it may elicit its immunosuppressive actions. Heat shock proteins may to play other roles in the immune response. They appear to be involved in the binding and stabilization of immunoglobulin heavy chains before binding of light chains (342). They may also interact with the processing and presentation of MHC class II molecules (343). Indeed, there are several intriguing possibilities by which DSG may exert its immunosuppressive actions.

Animal Studies with Deoxysperguralin in Transplantation of the Small Bowel and Other Organs

In animal models, DSG has been shown to prolong cardiac, renal hepatic, pancreatic and thyroid allografts with a wide range of doses (291). One of the earliest animal studies demonstrated that doses of 6.25 mg/kg/d for 10, 20 and 30 days were able to prolong rat skin graft survival in a dose-dependent manner (344). In rodents, daily administration of 1 to 5 mg/kg/d, intraperitoneal, (i.p.), for 2 to 8 weeks can effectively prolong the survival of cardiac and pancreaticoduodenal allografts (336,345,346). 1.25 or 5 mg/kg/d, i.p., for 10 days has been also shown to prolong thyroid allograft survival in mice (347) while 2 mg/kg q2 to 3 daily over a 2 week perioperative courese permits survival of pancreatic islets in rats (348). In larger animals, much lower doses appear effective for rejection prophylaxis. Canine renal allografts treated with prednisone and DSG at 0.8 mg/kg/d, i.v., for 10 days show prolonged survival with little toxicity (349). Two to five times higher doses are required for rescue therapy, however (350). Similar efficacy is seen with this dose in liver transplanted pigs (351).

As discussed above, DSG inhibits T-cell proliferation by a mechanism distinct from that of CsA. Subsequently, investigators have demonstrated the synergistic immunosuppressive actions of these two

drugs. Gannedahl *et al* demonstrated a 3-fold increase in graft survival in a rat cardiac allograft model when DSG (2 mg/kg/d, i.p. for 9 days) was coadministered with CsA (10 mg/kg/d, p.o., for 9 days) as compared to when these agents were administered individually (352,353).

With its known effect on inhibiting both cellular and humoral immunity, DSG has been investigated in xenografting (340,354). Suzuki *et al* demonstrated significantly prolonged hamster to rat heart allograft survival to 4.6 days (controls, 3.3 days) using a dose of 5 mg/kg/d using a constant i.p. infusion for 8 days (354). When recipients underwent a splenectomy in addition to the drug infusion, graft survival was further extended to 10.9 days. They had demonstrated a significant increase in the B-cell population in recipients after xenografting and thus, removal of the spleen facilitated a delay antibody production against donor cells.

Limited studies have investigated the effect of DSG on SBT. However, Tanaka *et al* were able to demonstrate the ability of DSG, administered for 10 days post SBT (5 mg/kg/d, i.p.) to prevent the development of cutaneous or lethal GVHD in a one-way GVHD rat model (355). More recently, in a swine SBT model, 3 mg/kg/d, i.v., for one week followed by maintenance therapy at half this dose, allowed for significant prolongation of allograft survival, although it was not as efficacious as immunosuppression with CsA (356).

Clinical Experiences with Deoxyspergualin in Organ Transplantation

In a clinical series of renal transplant patients, DSG was able to reverse allograft acute rejection in 13 out of 15 patients (357). Okubo *et al* looked at DSG as rescue therapy for steroid-resistant rejection of renal transplants (358). They found it to be as equally effective as conventional treatment with OKT3, with a significantly lower incidence of adverse effects, and proposed that it may be a worthwhile alternative for those patients who are OKT3 resistant. Similar results have been reported by others (359,360). Dosages of 3 to 5 mg/kg/d for 7 days have been recommended (359).

Success has also been achieved in pancreatic islet transplantation. Unlike CsA, FK506 and prednisone, DSG does not appear to be associated with altered beta-cell function or glucose utilization in

rats (361,362). This lack of diabetogenicity led Gores *et al* to utilized DSG (4 mg/kg/d for 10 days) with CsA in clinical islet transplantation in two patients. One has had a significant reduction of insulin need and the other has remained independent of insulin for 4 months at the time of publication (363).

To date, there have been no published studies of DSG being used in animal or human SBT. However, its successes in animal models of other vascularized organs and in clinical renal and islet transplantation, its relatively low toxic profile, and its alternate mechanism of action compared to more conventional immunosuppressive agents makes it an attractive alternative to be investigated for use in SBT.

Adverse Effects

In rats, side effects of DSG include weight loss, bone marrow toxicity and infectious complications (291). Doses of 5 mg/kg/d, i.m., were shown by Jindal *et al* to be toxic to rats after 1-2 weeks of treatment, with marked weight loss, patchy hair loss, lethargy, diarrhea, nosebleeds, sepsis and evidence of respiratory distress (362). Mice treated with 5 mg/kg/d, i.p., for 2 or more weeks also develop significant but reversible weight loss as well as anemia, leukopenia and alterations of hematopoietic and lymphoid tissues (345,347,364). Larger animals such as dogs and swine appear to be more sensitive to DSG with the development of severe gastrointestinal disturbances such as diarrhea, bleeding, and emaciation, even in a minimally immunosuppressive dose of 0.6 to 1.8 mg/kg/d. (349,351,365). However, renal transplanted monkeys treated with 2 mg/kg/d and 6 mg/kg/d, s.c., developed only skin rashes, appetite loss and slight elevation in BUN (without a concomitant rise in creatinine); these effects disappeared upon cessation of the drug (366).

The toxic effects of DSG in humans have been well described in a large series of renal transplant patients being treated for rejection (359,360). Doses of 3 to 5 mg/kg/d were given over 5 to 10 days. Adverse effects were generally dose-dependent. Subjective complaints of facial numbness, G.I. disturbances (including abdominal distention, upper abdominal discomfort, epigastralgia and heartburn) and headache occurred in 21-35 % of patients. Infectious complication of the respiratory tract, urinary tract, oral cavity and skin occurred in 8-23% of patients. Infections with CMV were not seen. The most

significant effects were alterations of the hematologic system, occurring in 29-75% of patients. This included the development of leukopenia in 29-75%, thrombocytopenia in 4-25%, and erythrocytopenia in 8-36%. It took and average of 9.5 days to reach the lowest white cell counts from the initiation of treatment. In severe cases of leukopenia, effective therapy (without discontinuation of the DSG) was obtained with administration of granulocyte colony-stimulating factor (G-CSF). None of these adverse effects were severe enough to warrant discontinuation of the drug.

RS61443 (MYCOPHENOLATE MOFETIL)

Mechanisms of Action

Mycophenolate mofetil (RS61443) is a semisynthetic prodrug, a derivative of the fungal antibiotic mycophenolic acid (MPA), isolated from the mold *Penicillin glaucum* (357). After oral administration of RS61443, it is rapidly hydrolyzed to release MPA, which selectively inhibits two enzymes required for *de novo* biosynthesis of guanosine monophosphate: inosine 5'-monophosphate dehydrogenase (IMPDH), the rate-controlling enzyme in the *de novo* biosynthesis of GTP, and guanosine monophosphate synthetase (367-371). Unlike other cells which have a salvage pathway for purine synthesis, T- and B-cells depend on this *de novo* pathway. Thus, in the presence of RS61443, which selectively depresses *de novo* purine synthesis and therefore decreases intracellular guanine nucleotide pools, T- and B-lymphocytes can no longer proliferate in response to an antigenic stimulus.

This reduction of intracellular GTP levels in T- and B-cells limits the availability of nucleotides for GTP protein functions in signal transduction and the biosynthesis of glycoproteins and cell surface receptors, thereby impairing the cellular activation process. This is consistent with the observed unresponsiveness of T- and B-cells upon mitogenic activation (372). These *in vitro* observations include inhibition of proliferation of T- and B- cells and suppression of the B-cell memory responses.

Inhibited biosynthesis of glycoproteins appears to occur at the glycosylation phase. Allison *et al* were able to demonstrate *in vitro* that mycophenolic acid is able to inhibit the glycosylation of adhesion

molecules (373). The depletion of GTP pools prevents the formation of GDP-fucose and GDP-mannose, intermediates in the transfer of fucose and mannose to dolichol phosphate-linked oligosaccharides and then to proteins. Subsequently, this inhibits the binding of activated human lymphocytes to activated human endothelial cells. This interaction is required in the rejection response to allow leukocytes to "stick" to and subsequently migrate between the endothelial cells into the perivascular connective tissue. It is possible that MPA may also inhibit the interaction between complementary adhesion molecules acting in the immune response. An example would be the ICAM-1 and its complementary ligand, LFA-1. When the interaction of these two molecules is block by anti-ICAM-1 and anti-LFA-1 antibodies, prolonged survival of mice receiving cardiac allograft is observed, with no further immunosuppression needed (374). Any similar negative effect of MPA on the interaction of these molecules would enhance its immunosuppressive properties

Animal Studies in Transplantation of the Small Bowel and Other Organs

Early animal studies using RS61443 in organ transplantation demonstrated that in rat heart allograft models, doses of 20 to 40 mg/kg/d, p.o., for 50 days, could produce significant graft survival in a dose-dependent fashion (369). Moreover, it was also effective in reversing advanced allograft rejection, in the same model, at doses of 30 mg/kg/d. In a canine renal allograft model, 40 mg/kg/d , p.o., prolonged graft survival to 36 days (controls, 8.1 days), however, this dose produced severe gastrointestinal effects in the animals (368). When the dose was halved and combined with low dose CsA and methylprednisolone, allograft survival was prolonged to an average of 122 days, with 6 of 16 dogs continuing to survive over 150 days. A marked reduction in toxicity was also seen. Use of CsA plus methylprednisolone, alone, did not induce any survival advantage. Similar results were obtained in a canine liver allograft model using combined doses of RS61443 of 20 mg/kg/d and CsA at 5 to 10 mg/kg/d, with markedly prolonged graft survival and minimal side effects (375).

Further demonstrations of the efficacy of RS61443 in combination with other agents have been published. Combined with Brequinar sodium, a new immunosuppressive agent with a complementary

action to RS61443 (see below), significant prolongation of rat heart allograft survival has been demonstrated with no adverse effects seen (376).

With these promising results in transplantation of other organs, RS61443 has been investigated for use in SBT. When used alone, prolonged administration of 25 mg/kg/d, i.p., or a 6 day course of 30 mg/kg/d, p.o., effectively prevents GVHD while being unable to suppress HVG rejection in one-way and two-way rat SBT models (377,378). D'Alessandro *et al* found that when administered with fully therapeutic doses of CsA and methylprednisolone, 20 mg/kg/d of RS61443 markedly prolongs survival of allogeneic 150 cm segmental allografts in dogs, whereas either agent alone is ineffective (379,380). This differs from the results seen in canine liver and renal transplantation, and rat cardiac allografts, where combination with **subtherapeutic** doses of CsA successfully prolong allograft survival (368,369,375). This may reflect the greater immunogenicity of small bowel allografts as compared to other organs. Therefore, the combined action of RS61443 and CsA or FK506 appears to be complementary rather than synergistic, as no survival advantage of small bowel allografts is obtained when combining RS61443 with subtherapeutic doses of CsA, although the recipients have a better clinical course, with better eating and weight gain patterns than with either agent alone (380,381).

Clinical Experiences

One of the first clinical trials with RS61443 utilized doses of 100-3500 mg/d, p.o., in combination with low dose CsA (5-10 mg/kg), ALG and methylprednisolone to treat 44 primary renal allograft recipients (382). Overall patient and graft survival were 100% and 95%, respectively, after 18 months. It was generally well tolerated in all dose groups. Hemorrhagic gastritis was the one major adverse effect noted in all groups, requiring drug discontinuation. Dosage reduction was required in 5 patients because of persistent nausea and vomiting in two, diarrhea in one, leukopenia in one, and prolonged elevation of liver function studies after hepatitis C infection in one. Nephrotoxicity was not seen. A similar European multicenter study was carried out utilizing 400-2000 mg/d of RS61443 and 10-15 mg/kg/d of CsA, maintaining a plasma trough level of 150-250 μ/L (383). They, however, found no benefit of RS61443.

In fact, patients receiving both drugs more often experienced more severe rejection responses and had a markedly increase frequency of infections, especially bacterial. Other adverse effects, however, were minor. The authors feel that the doses used may have been subtherapeutic, but caution that at high doses, side effects and infectious complications are more prevalent.

RS61443 has also been investigated in a multicenter study as rescue therapy for refractory renal allograft rejection using 2000-3000 mg/d (384). It was most efficacious when initiated in early rejection, when creatinine levels had not yet exceeded 4 mg percent, with a response rate of 79%. This agent was equally successful when used for treatment of refractory liver allograft rejection (385). Of 23 patients, 21 responded with either resolution of rejection or at least improvement of liver function. However, two of these patients required cessation of therapy for persistent cholestasis, two required retransplantation (one for chronic rejection and one for acute rejection), and one died from CMV/HSV/candida infection. There were four patients with chronic rejection entered into the study, but none responded to the drug. The use of RS61443 in chronic rejection thus appears less promising.

RS61443 has also proved effective in the management of cardiac allograft rejection (386). Thirty patients, 26 on azathioprine and 4 on cyclophosphamide, were treated with doses of 500 mg daily to 1500 mg twice a day. There was a 67% overall resolution of rejection. The least successful responses were seen in the lowest dose group (500 mg daily), a response rate comparable to that seen with azathioprine alone.

Adverse Effects

In animal studies, RS61443-induced gastrointestinal effects, including gastritis, diarrhea and anorexia, appear to be the most prominent, and are dose-related (368). Except for a slight elevation in alkaline phosphatase, no hematologic abnormalities, hepatotoxicities or nephrotoxicities have been noted.

In human studies, as discussed above, hemorrhagic gastritis can occur along with other gastrointestinal symptoms such as nausea and vomiting, sometimes requiring cessation of the drug (382,384,386). Other less common side effects are the development of pancreatitis and CMV colitis

(384). Some reports indicate an increased frequency of infections (383). As in animals, no significant hepatotoxic or nephrotoxic effects are seen (386). More significantly, unlike CsA and FK506 which are associated with the development of PTLD, RS61443 may result in fewer immunosuppressive-related malignancies. This is the result of its specific target to lymphocytes, with no substantial inhibition of other cell lines. Subsequently, as most PTLDs develop from Epstein-Barr virus (EBV)-transformed B-lymphocytes, RS61443 can actually block the proliferation of these EBV-transformed cell lines (387).

NEWER IMMUNOSUPPRESSIVE AGENTS

BREQUINAR SODIUM

The search for additional, more powerful and less toxic immunosuppressive agents continues in fervor in the pharmaceutical industry. One of the more recently developed agents is Brequinar sodium (BQR), a synthetic compound (quinoline carboxylic acid analogue) originally developed as an antitumor agent, but found to have powerful immunosuppressive actions (291,370,388). The action of BQR parallels that of RS61443, in that it inhibits the *de novo* pathway of pyrimidine synthesis via a noncompetitive inhibition of the enzyme dihydroorotate dehydrogenase. Most cells have a salvage pathway by which to synthesize uridine and cytidine, however, lymphocytes are dependent on this pathway for the synthesis of RNA and DNA. Thus, akin to RS61443, lymphocytes are highly sensitive to the actions of BQR, rendering it more specific, and thereby less toxic to other human cell lines.

Like RS61443, BQR acts later in the rejection response when activated cells respond to antigen challenge and begin to synthesize DNA for cell division. It is highly effective in suppression of both humoral and cell-mediated immunity. Its potent inhibition of antibody formation makes it attractive for treatment of accelerated allograft rejection in already sensitized recipients as well as for use in xenotransplantation (388).

Glycosylation of adhesion molecules was discussed above in relation to inhibition by RS61443. BQR can also inhibit glycosylation by its depletion of UTP, required for the formation of UDP derivatives of

glucose, galactose and the corresponding amines (373). Transfer of one of these units of UDP-GlcNac to dolicholphosphate is the first step in glycosylation.

In vitro studies by Jaffee *et al* clearly demonstrate these effects (389). BQR is able to suppress human MLR more effectively than either CsA or azathioprine. It is also more potent than CsA in inhibition of T-cell mediated augmented delayed-type hypersensitivity, similar to that seen with clinically presensitized patients. In addition, it is able to inhibit the development of cytotoxic T-cells and suppress *in vivo* antibody response to foreign antigens. Of specific interest is the observation of synergistic activity when suboptimal doses of BQR and CsA are combined in suppression of the immune response in a mouse contact sensitivity model.

Also of specific interest is the combined effects of BQR with RS61443. RS61443 depletes GTP while BQR depletes UTP. Their effects would at least be expected to be additive at several stages of the immune response (370). Firstly, at the glycosylation stage. Secondly, RS61443 has also been shown to deplete 5-phosphoribosyl-1-pyrophosphate (PRPP), which is also required for the synthesis of UTP, hence reinforcing BQR's effects. Thirdly, BQR acts to deplete dTTP which is required for the reduction of GTP to dGDP, hence reinforcing RS61443's actions.

Animal Studies in Small Bowel and Other Organ Transplantation

Initial studies of BQR in allografting showed it to be effective in prolonging heart, liver and kidney allograft survival in rats (390). Treatment with 12 mg/kg, p.o., three times weekly, produced median survivals of these organs of 45, 91 and greater than 100 days, respectively. Further studies demonstrated tolerance-induction acceptance of donor-specific hearts by long-term surviving liver recipients (391). BQR's ability to reverse ongoing rejection was demonstrated by a three day course started 6 days post-transplantation (after the onset of moderate acute rejection), suggesting its potential suitability for rescue therapy. Bone marrow suppression and diarrhea led to the demise of some of these animals, however.

Further studies demonstrated BQR suppression of IgM and IgG antibody production in rats transplanted with a cardiac allografts, 7 days after having become presensitized with a skin graft from the

same donor (392). The greatest effect was seen with therapy given during both the sensitization and effector phases, with significant prolongation of allograft survival compared to treatment during the effector phase alone.

As described earlier, the synergistic combination of RS61443 with BQR creates a potent immunosuppressive effect (376). Similarly, synergism between BQR and CsA has been demonstrated in a rat cardiac transplant model (393,394). Combined low doses of BQR with subtherapeutic doses of CsA resulted in significantly prolonged allograft survival compared to controls. Neither of these drugs, at these low doses, were effective when given alone. Interestingly, CsA was found to cause an increase in the BQR levels, such that some of the observed synergism may be a reflection of these elevated levels.

This synergism has been shown to prolong graft survival in a one-way rejection model of SBT in the rat (395). Combined doses of BQR, 2.5 mg/kg every third day, via the proximal enterostomy, and CsA, 0.5 mg/kg/d, i.m., permitted a mean graft survival of 54 days before rejection ensued, with 2 of 5 animals surviving long-term without any sign of rejection. Mean graft survival in nonimmunosuppressed animals was only 8 days; and in rats treated with BQR or CsA alone, mean graft survival was 10.4 and 11.8 days, respectively. No toxicities were noted, however, when the BQR dose was increased to 5 mg/kg, 4 of the 7 animals died from infectious complications.

Extension of these findings has led to the investigation of the combination of BQR with dual immunosuppression of RAPA and CsA, two agents which, as discussed earlier (in the section on RAPA), are also synergistic (319,323,324). Kahan and Stepkowski *et al* (396,397) demonstrated that a low dose combination of CsA (0.5 mg/kg/d, i.v.) and RAPA (0.01 mg/kg/d, i.v.) significantly prolonged cardiac allograft survival in rats by 5-fold, to 35 days, compared to untreated controls, whereas when administered alone, neither of these drugs prolonged graft survival. Addition of BQR at low doses of 0.5 to 2.0 mg/kg/d, p.o., further prolonged graft survival up to 116.8 days. Pharmacological analysis showed this to be a synergistic response. This synergistic action may be explained by the different effects of the three drugs on the biochemical pathways of the rejection response. CsA inhibits the generation of IL-2

regulating early activation genes during the G_0 phase of the cell cycle. RAPA induces cell cycle arrest in the G_1 phase and BQR inhibits nucleotide synthesis, hence blocking cells in the S-phase (397).

Clinical Studies

No human studies utilizing BQR have yet been published. Makowka *et al* , however, published preliminary studies of cardiac transplantation in primates (388). Allograft recipients were treated with BQR, 2 or 4 mg/kg, p.o., 3 times per week, with or without low dose CsA, 2 mg/kg/d, i.m. This combination of BQR and CsA significantly prolonged median allograft survival to 38 days compared to untreated controls and those treated with CsA only.

Adverse Effects

In animals, bone marrow suppression, diarrhea and infectious complications are seen at higher doses of BQR (up to 12 mg/kg, 3 times per week) as described above (391,395). Toxicologic studies have been performed by Barnes *et al* and showed that at 5 mg/kg/d, significant toxicities are associated with tissues/organs that have a high rate of cell replication (398). These include bone marrow suppression with anemia and leukopenia, cellular depression of lymphoid elements (thymus and spleen) and gastrointestinal toxicity manifested in depressed epithelial growth and intestinal mucosal atrophy and degeneration. Six percent of the rats died. Moreover, when CsA was co-administered (10 mg/kg/d), these toxic effects were more severe and 69% died. When BQR was administered at 5 mg/kg every alternate day, no toxicities were observed, with or without the addition of CsA.

LEFLUNOMIDE

Leflunomide is an isoxazol derivative with an ability to suppress both T-cell and B-cell activity, likely related to an inhibition of lymphocyte tyrosine kinase activity. It is hypothesized to prevent T-cell proliferation primarily by inhibiting IL-2 signal transduction, resembling RAPA in its mechanism of action (399). Leflunomide has been shown to prevent and reverse (rescue) rejection of rat cardiac renal and skin allografts with a low incidence of toxic effects (400-402).

In rat cardiac transplant models, Leflunomide, 5 mg/kg/d, p.o., for 2 weeks, can prolong allograft survival 4-fold to 28 days (400). The addition of low-dose CsA further prolongs survival in a synergistic fashion, to 33 days, significantly longer than with BQR or CsA alone.

In rat SBT models, Leflunomide, 5 mg/kg/d for 10 days followed by every alternate day for 2 months, results markedly long-term survival of greater than 150 days, with the development of partial tolerance, as demonstrated by donor-specific skin grafting 100 days post-transplantation (403). Another study of SBT in the rat utilized doses of 5 mg/kg/d, p.o., for only 1 to 4 weeks, and found significant prolongation of survival in all study groups, especially with the longest duration of treatment, with a MST of 34 days compared to 10 days in untreated controls (404). The i.v. form of Leflunomide, A77, at 5 mg/kg/d prolonged survival to 39 days with only 2 weeks of treatment. Furthermore, when the same dose of A77 was utilized in one-way HVG rejection and GVHD models, survival was prolonged to greater than 150 days in both, revealing this agent's efficacy in inhibiting both responses.

In view of these results and Leflunomide's relatively low toxicity profile, it is indeed an agent to be more closely studied as a potential candidate for use in SBT.

MULTIDRUG REGIMENS

Indeed, the problems encountered in SBT appear to be centered around the development of adequate immunosuppressive regimens due to the high immunocompetency of the allografts and unfortunate high rate of graft rejection observed. It is obvious that the arsenal of immunosuppressive agents available for clinical transplantation is enormous and powerful. However, each agent is not without its significant toxicities. Our current understanding of the cascades of events involved in allograft rejection and the corresponding roles of these various immunosuppressives now permits the rational development of immunosuppressive regimens utilizing combinations of agents with complementary and synergistic actions. By more specifically targeting various steps in the immune response, lower doses of these agents may be applied, with a concomitant reduction in their individual toxicities. As discussed in the preceding

sections on various immunosuppressive agents, such rationale is already being applied in the development of such regimens, and appears to hold great promise. Some of the more intensely studied combinations have already been discussed, but it is beyond the scope of this writing to illustrate all of the multidrug regimens currently under investigation. Thus, advances in SBT do not necessarily require the development of more powerful agents, but rather, therapy resulting in less toxicity to the recipient, and with the least effects on allograft function.

OTHER METHODS OF IMMUNOMODULATION AND TOLERANCE INDUCTION FOR SBT

MONOCLONAL ANTIBODIES (MAbs)

Polyclonal antibodies include rabbit or equine antilymphocyte globulin (ALG) and antithymocyte globulin (ATG). They have been utilized for years, in combination with other immunosuppressives, for induction therapy or treatment of rejection responses, in efforts to utilize lower and less toxic doses of agents such as CsA. They act by opsinizing T-cells, leading to their removal from the circulation, thereby obviating T-cell mediated attack on the allograft (141). However, this action is relatively nonspecific. Adverse effects are common and include fever, leukopenia, anaphylaxis, serum sickness, rash pruritis, hypertension, gastrointestinal upset and increased risk of infection (226). Aside from their use in conventional immunosuppressive regimens, these agents have applicability to SBT in pretreatment of the donor. As discussed above in the section on "reduction of allograft immunocompetent cell load", pretreatment of the donor with ALS has been shown to deplete the lymphoid tissue of the allograft, preventing the occurrence of GVHD in a rat SBT model (159,175,176).

A significant advancement in the evolution of these agents has been the development of antibodies directed specifically towards structural components of immunocompetent cells or other molecules that mediate or sustain the rejection response: monoclonal antibodies (MAbs). Over the past years, a plethora of such agents have been developed, and some excellent reviews have been published (141,291). A brief outline will be presented here.

The prototype monoclonal antibody, mouse OKT3, complexes with the CD3 receptor on the surface of T-cells and blocks their function. It is most commonly used for the treatment of rejection episodes, but can also be used for induction therapy. However, it has many serious limitations (141,226). Firstly, it elicits a profound production of lymphokines, especially tumor necrosis factor (TNF) and IL-2, resulting in a characteristic first dose response of chills, fever, myalgias, dyspnea, chest pain, and possibly severe pulmonary edema. Secondly, approximately 30% of patients develop anti-idiotypic antibodies, precluding subsequent courses of OKT3 should rejection recur. Thirdly, the therapeutic effect of this agent may be delayed for as long as 7 days, and there is a frequent rebound of rejection episodes upon completion of the therapeutic course. Finally, OKT3 is associated with an increased risk of viral infections (especially CMV, which can be seen in up to 40% of patients) and development of lymphomas and other malignancies. This latter complication is most significant when OKT3 is used in the pediatric transplant population.

Other newer MAbs have been developed recently (291). BMA031 and T10B9 are anti-T-cell receptor (TCR) antibodies which have shown some promise in being at least as efficacious as CsA in preventing and reversing rejection responses. T10B9, specifically, is an IgM antibody and therefore lacks binding to monocyte F_c receptors which are responsible for lymphokine mediated toxicity. Other MAbs directed against the IL-2 receptor can inhibit the efferent phase of the immune response by eliminating or suppressing the function and expansion of T-cells responding to alloantigen. Clinical trials utilizing these agents with more conventional therapy (CsA, ATG) have shown mixed results of either equal or slightly improved efficacy in preventing rejection. Side effects appear to be minimal. Anti-CD4 MAbs, directed against $CD4^+$ cells, have also shown some promise, with fewer side effects. They act by inducing a state of lymphocyte hypo-responsiveness to donor alloantigens. In a rat SBT model, Bowles *et al* have demonstrated that anti-CD4 MAbs can significantly delay, but not prevent, rejection with pre- and post-transplant treatment of the recipient (405). MAbs against lymphokines (such as TNF), act by down-regulating the immune response and also appear efficacious, with few side effects.

As discussed earlier, adhesion molecules appear to be important in the immune response, allowing interaction between host immune cells and donor graft cells. Intracellular adhesion molecule-1 (ICAM-1) is found on the cell surface of most cells, including vascular endothelial cells, and mediates adhesion with immune cells through the leukocyte function-associated molecule-1 (LFA-1) (141,142). Use of combined therapy with anti-ICAM-1 and anti-LFA-1 MAbs shows some promise. With no other immunosuppression required, these have been shown to induce graft acceptance and host tolerance in animal models of organ transplantation and in some initial clinical studies (142,374).

MAbs targeted against antigens on donors cells such as MHC class I and/or class II antigens have also been shown to be powerful agents when administered as either donor pretreatment or host therapy (291). To investigate the use of these agents in SBT, Stangl *et al* (406) used two different monoclonal antibodies directed specifically against rat class II antigen, and macrophages and dendritic cells, respectively. Small bowel allograft pre-treatment with these agents revealed a statistically prolonged graft and recipient survival. Correspondingly, *in vitro* mixed lymphocyte culture studies also show the efficacy of these monoclonal antibodies in preventing an immune response.

Unfortunately, most patients treated with these MAbs eventually develop anti-mouse antibodies, resulting in decreasing levels of agent and attenuated effects. Recently, there have been attempts to construct hybrid human-mouse MAbs that combine human-type constant domains with mouse-derived epitope-specific domains (141). Therefore, fewer foreign amino acid sequences are presented to the host. However clinically, mixed results have been observed and some investigators feel that the more "humanized" forms have reduced affinity for antigen epitopes, although side effects are minimized.

DONOR SPECIFIC TRANSFUSION (DST)

Pre-transplantation blood transfusion of donor blood to the recipient has been shown to suppress rejection of murine skin, heart and renal allografts, and has been employed clinically in renal transplantation (407-409). The mechanism appears to involve modification of the immune response

through the induction of splenic suppressor cells, and the generation of anti-idiotypic antibodies. This induces a state of specific "hyporesponsiveness" toward the donor (407). The concurrent administration of an immunosuppressive agent such as CsA or RAPA appears to be necessary and synergistic. DST alone has been frequently reported to be detrimental to allograft survival, probably by causing a slight sensitization towards the donor.

In the rat model of SBT, Fecteau *et al* have shown that significant allograft survival is not seen with one DST given 24 hours prior to transplantation, with coadministration of low-dose CsA (410). In contrast, a similar regimen of pre-transplant DST combined with low-dose CsA was sufficient in significantly prolonging allograft survival in a rat cardiac transplantation model as compared to CsA or DST alone (407). The authors hypothesize that the strong immunogenicity of the small bowel, as compared to other vascularized organs, may induce an immunological reaction too strong for one DST to counteract. Similar results have been observed by other investigators (411,412).

Interestingly, however, DST given 24 hours pre-transplant, directly into the portal venous system, in combination with a short course of CsA does prolong rat SBT allograft survival (MST of 70 days compared to 9 and 14 days, respectively, with DST and CsA alone) (413). This may involve a process of donor-specific antigen processing to result in inert circulating immune complexes in a mechanism similar to that proposed for the induction of tolerance by liver allografts (414). Conversely, low-dose FK506 does not induce immunologic tolerance associated with intraportal DST, and in fact, appears to abrogate any beneficial effect (413).

Further studies have combined DST with pre-transplantation allograft irradiation and a short course of CsA, demonstrating a significant prolongation of SBT survival (415). DST alone does not appear to be able to control GVHD or rejection, again, probably because of the large content of lymphoid tissue in the graft intestine. Allograft irradiation alone has been shown to be inefficient in controlling HVG rejection responses (as discussed earlier). The combined effects, however, probably employing similar synergism as seen between DST and CsA in other allograft models, appear sufficient to ensure prolonged survival.

However, the clinical applicability of this process is questionable, especially in consideration of potential long-term intestinal dysfunction secondary to irradiation (174).

SIMULTANEOUS LIVER TRANSPLANTATION

Liver transplantation has been long-known for its protective effect in the prevention of rejection of other organ allografts (416,417). It appears to be able to induce a state of donor-specific tolerance. The mechanism of this is still not well understood, but may be related to an ability to release MHC-I antigens in a soluble form, rendering them susceptible to combine with cytotoxic antibodies to create inert circulating immune complexes. The co-transplanted liver may also be able to specifically absorb these cytotoxic antibodies. At the same time, the liver can trap donor-reactive lymphocytes, specifically cytotoxic T-cells reactive against donor class I antigens, and deplete them from the recirculating lymphocyte pool (414,418).

The ability of liver co-transplantation to improve survival of clinical renal allografts (419) has led investigators to look at its effects on SBT. Indeed the first successful SBT was performed with a concomitant liver allograft from the same donor as described earlier (126). However, the clinical outcomes of large series of isolated small bowel (SB) transplants compared to small bowel-liver allotransplantation (SB/L) are not as clear cut as seen with renal-liver transplants.

The largest recently reported series, from Pittsburgh, appears to show no significant graft survival of SB/L over SB only transplantation (133). One-year patient and graft survival rates for SB/L versus SB only transplant cases were 76% and 73% versus 91% and 70%. However, other confounding factors may make the interpretation of these results difficult. For example, 77% (20 of 26) of SB/L cases were pediatric patients while 32% (7 of 22) of SB only cases were children. Any survival disadvantage which children may be susceptible to may affect these results. For example, many of these children are on prolonged TPN prior to transplantation which may induce serious metabolic and hepatic abnormalities that can have negative effects on their overall post transplantation clinical course.

In contrast, another series from London, Ontario reports that intestinal rejection appears to be uncommon with simultaneous liver grafting. However, these patients were more prone to the development of sepsis or lymphomas (127). More complete series with longer follow-ups will be necessary to more conclusively determine the potential benefit of simultaneous liver transplantation.

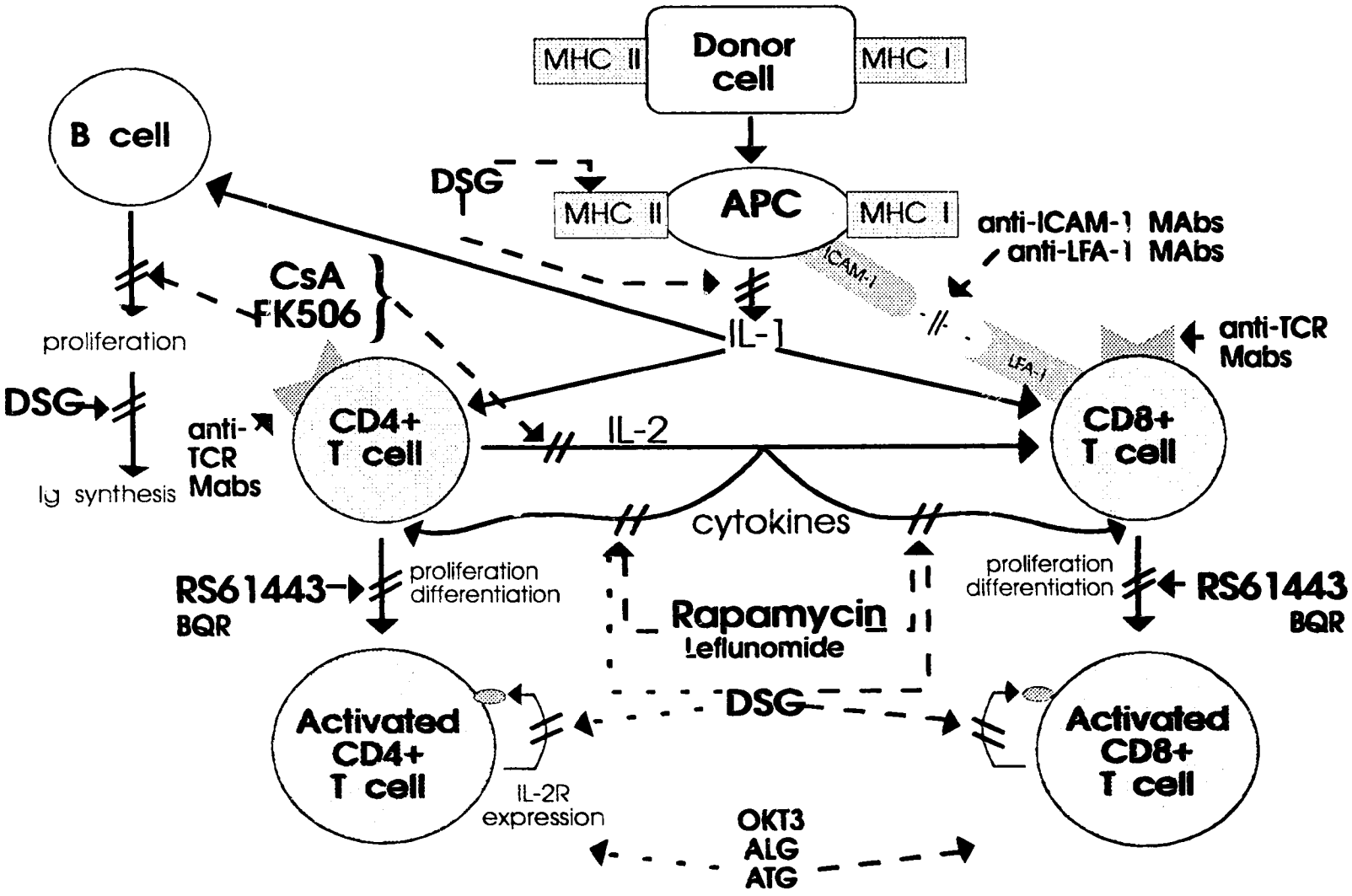
CONCLUSION

SBS certainly represents a challenge to the medical and surgical community. With the innumerable problems associated with a short gut and the need for long-term TPN, there is a strong need for the development of a cure for SBS: small bowel transplantation. Despite early recognition of the technical feasibility of SBT, experimental and clinical successes have been greatly hindered by the inability to develop adequate immunosuppressive protocols. Many promising agents have been shown to be effective in animal and clinical studies, but the toxicities associated with these powerful drugs limit their use. Cyclosporin, one of the first immunosuppressives to be used in the modern era of organ transplantation, has shown some promise, and indeed has been utilized in the first successful isolated SB and SB/L transplants. The newer era of immunosuppression has led to the development of FK506, rapamycin, 15-deoxyspergualin and RS61443. These more powerful agents, each with unique mechanisms of action and unique toxic effects, have also shown some promise in both experimental and clinical studies of SBT. More so, the potential for rational development of multiagent regimens may provide even stronger immunosuppression, with a profound reduction of drug toxicites. The addition of other modalities and agents to enhance immunosuppression, such as the use of pre-and post-transplant monoclonal antibodies, donor-specific transfusions and simultaneous liver transplantation, especially in cases of SBS associated with liver dysfunction or failure, may also add to our ever growing arsenal of methods to prevent allograft rejection. A much greater understanding of the immunological phenomena occurring in SBT and the actions of these agents on the complexity of the immune response seen in humans will be needed before further clinical advancements can be truly made.

However, acceptance of a small bowel allograft by the recipient is not the only criteria for successful SBT. In addition, the graft must be functional with respect to nutrient absorption in order to support nutrition and growth of the host. As discussed earlier, in the section on "cyclosporin", the transplant process itself, by virtue of denervation, disruption of lymphatics, and other less well defined mechanisms, can have profound effects on allograft function (242-250). Thus, any further adverse effects induced by the immunosuppressive agents employed should be minimized.

Few studies have looked at the effects of cyclosporin and the newer agents being investigated for SBT on nutrition and small bowel function, particularly as it relates to growth which is important when being considered for use in the pediatric SBS population. In order to obviate the confounding effects of the transplantation process, our laboratory has undertaken studies to investigate the effects of these agents on growth, nutrition and small bowel function, both *in vivo* and *in vitro*, in normal juvenile rats.

Figure II-1
Mechanisms of Immunosuppression



REFERENCES

1. Wright JK, Jr. Short gut syndrome: Options for management. Compr Ther 1992; 18(8): 5-8.

2. Nightengale JMD, Lennard-Jones JE. The short bowel syndrome: What's new and old. Dig Dis 1993; 11: 12-31.

3. Sigalet DL, Thomson ABR. Consequences of small bowel resection. Med N Amer 1988; 18: 3423-32.

4. Flint JM. The effect of extensive resections of the small intestine. Bull Johns Hopkins Hosp 1912: 23: 127-44.

5. Hammond HE. Massive resection of the small intestine. An analysis of 257 collected cases. Surg Gyne Obstet 1935; 61: 693-705.

6. Hiyama DT. The current role of small-bowel transplantation in intestinal failure. Nutr Clin Prac 1993; 8(1): 5-11.

7. Karasov WH, Diamond JM. Adaptation of intestinal nutrient transport. In: Johnson LR, editor. Physiology of the gastrointestinal tract. 2nd ed. New York: Raven press 1987: 1489-97.

8. Spiller RC, Trotman IF, Higgins BE, Ghatei MA, Grimble GK, Lee YC, *et al.* The ileal brake-inhibition of jejunal motility after ileal fat perfusion in man. Gut 1984; 25: 365-74.

9. Pironi L, Stanghellini V, Miglioli M, Corinaldesi R, De Giorgio R, Ruggeri E, *et al.* Fat-induced ileal brake in humans: a dose-dependent phenomenom correlated to the plasma levels of peptide YY. Gastroenterol 1993; 105: 733-9.

10. Spiller RC, Trotman FF, Adrian TE, Bloom SR, Misiewicz JJ, Silk DBA. Further characteization of the 'ileal brake reflex in man--effect of ileal infusion of partial digests of fat, protein, and starch on jejunal motility and release of neurotensin, enteroglucagon and peptide YY. Gut 1988; 29: 1042-51.

11. Cosnes J, Gendre JP, Le Quintrec Y. Role of the ileocecal valve and site of intestinal resection in malabsorption after extensive small bowel resection. Digestion 1978; 18: 329-36.

12. Gazet JC. The surgical significance of the ileo-cecal junction. Ann R Coll Surg Engl 1968; 43: 19-38.

13. Mitchell JE, Breuer RI, Zuckerman L, Berlin J, Schilli R, Dunn JK. The colon influence ileal resection diarrhea. Dig Dis Sci 1980; 25: 33-41.

14. Wilmore DW. Factors correlating with a successful outcome following extensive intestinal resection in newborn infants. J Pediatr 1972; 80: 88-95.

15. Posthuma R, Moroz S, Friesen F. Extreme short bowel syndrome in an infant. J Ped Surg 1983; 18: 264-8.

16. Aaronson IA, Bowie MD, Cywes S, Jouw JH. Massive small bowel resection in a neonate. Arch Surg 1975; 110: 1485-90.

17. Ball MJ, Martin LW, Shubert WK, Partin J, Burke J. Massive small-bowel resection in an infant: Long-term management and intestinal adaptation. J Ped Surg 1973; 8: 197-204.

18. Grosfeld JL, F.J. Rescoria FJ, West KW. Short bowel syndrome in infancy and childhood: Analysis of survival in 60 patients. Am J Surg 1986; 151: 41-6.

19. Cooper A, Floyd TF, Ross AJ, III, Bishop HC, Templeton JM, Jr., Zeigler MM. Morbidity and mortality of short-bowel syndrome acquired in infancy: An update. J Ped Surg 1984; 19: 711-7.

20. Bell MJ, Martin LW, Schubert WK, Partin J, Burke J. Massive small-bowel resection in an infant: Long-term management and intestinal adaptation. J Ped Surg 1973; 8: 197-204.

21. Kurz R, Sauer H. Treatment and metabolic findings in extreme short-bowel syndrome with 11 cm jejunal remnant. J Ped Surg 1983; 18: 257-63.

22. Holt D, D. Easa D, Shim W, Suzuki M. Survival after massive small intestinal resection in a neonate. Am J Dis Child 1982; 136: 79-80.

23. Tepas JJ, MacLean WC, Kolbach S, Shermeta DW. Total management of short gut secondary to midgut volvulus without prolonged total parental alimentation. J Ped Surg 1978; 13: 622-6.

24. Ricour C, Duhamel J, Arnaud-Battandier F, Collard Y, Revillon Y, Nihoul-Fekete C. Enteral and parenteral nutrition in the short bowel syndrome in children. World J Surg 1985; 9(2): 310-5.

25. Georgeson KE, Breaux CW, Jr. Outcome and intestinal adaptation in neonatal short-bowel syndrome. J Ped Surg 1992; 27(3): 344-50.

26. Caniano DA, Starr J, Ginn-Pease ME. Extensive short-bowel syndrome in neonates: Outcome in the 1980s. Surgery 1989; 105(2): 119-24.

27. Weber TR, Tracy T, Connors RH. Short-bowel syndrome in Children: Quality of life in an era of improved survival. Arch Surg 1991; 126: 841-6.

28. Thomson ABR, Keelan M, Sigalet D, Fedorak R, Garg M, Clandin MT. Patterns, mechanisms and signals for intestinal adaptation. Dig Dis 1990; 8: 99-111.

29. Sigalet DL, Lees GM, Aherne F, Van Aerde JEE, Fedorak RN, Keelan M, Thomson ABR. The physiology of adaptation to small bowel resection in the pig: An integrated study of morphological and functional changes. J Ped Surg 1990; 25(6). 650-7.

30. Fedorak RN, Chang EB, Madara JL, Field M. Intestinal adaptation to diabetes: Altered Na-dependent nutrient absorption in streptozocin-treated chronically diabetic rats. J Clin Invest 1987; 79: 1571-8.

31. Philipott DJ, Butzner4 JD, Meddings JB. Regulation if intestinal glucose transport. Can J physiol Pharmacol 1992; 70: 1201-7.

32. Asitook K, Carlson S, Madara JD. Effect of phlorizin and sodium in glucose-elicited alterations in intestinal epithelia. Am J Pysiol 1990; 258: C77-C85.

33. Lund PK, Ulshen MH, Rountree DB, Selub SE, Buchan AM. Molecular biology of gastrointestinal peptides and growth factors: relevance to intestinal adaptation. Digestion 1990; 46(suppl 2): 66-73.

34. Iacono G, Carroccio A, Montalto G, Cavataio R, Lo Cascio M, Notarbatolo A. Extreme short bowel syndrome: A case for reviewing the guidelines for predicting survival. J Ped Gastroenterol Nutr 1993; 16(2): 216-9.

35. Chadwich VS, Modha K, Dowling RH. The mechanisms for hyperoxaluria in patients with ileal dysfunction. New Engl J Med 1973; 289: 172-6.

36. Howard L, Heaphey L, Fleming CR, Liniger L, Steiger E. Four years of North American registry home parenteral nutrition outcome data and their implications for patient management. J Parenter Enter Nutr 1991; 15: 384-93.

37. Hall RCW, Beresford TP. Psychiatric factors in the management of long-term hyperalimentation patients. Psych Med 1987; 5: 211-7.

38 Steiger JU, Srp F. Morbidity and mortality related to home parenteral nutrition in patients with gut failure. Am J Surg 1983; 145: 102-5.

39. Perl M, Hall RCW, Dudrick SJ, Englert DM, Sondra MS, Stickney K, Gardner ER. Psychological aspects of long-term home hyperalimentation. J Parenter Enter Nutr 1980; 4: 554-60.

40. Wateska LP, Sattler LL, Steiger E. Cost of a home parenteral nutrition program. JAMA 1980; 244: 2303-4.

41. Sigalet D, Kneteman NM, Thomson ABR. Small bowel transplantation: past, present and future. Dig Dis 1992; 10: 258-73.

42. Schraut WH. Current status of small-bowel transplantation. Gastroenterology 1988; 94: 525-38.

43. Galea MH, Holiday H, Carachi R, Kapila L. Short-bowel syndrome: A collective review. J. Ped Surg 1992; 27(5): 592-6.

44. Herfindal ET, Berstein LR, Kudzia K, Wong A. Survey of home nutritional support patients. J Parenter Enter Nutr 1989; 13: 255-61.

45. McCullough ML, Hsu N. Metabolic bone disease in home parenteral nutrition. J Am Diet Assoc 1987; 87: 915-20.

46. Seligman JU, Basi SS, Dietal M, Bayley TA, Khana RK. Metabolic bone disease in a patient on long-term parenteral nutrition: A case report and review of the literature. J Parenter Enter Nutr 1984; 8: 722-7.

47. Posthuma R, Trevenen CL. Liver disease in infants, receiving total parenteral nutrition. Pediatrics 1979; 63: 110-5.

48. Touloukian RJ, Seashore JH. Hepatic secretory obstruction with total parenteral nutrition in the infant. J Pediatr Surg 1975; 10: 353-60.

49. Lindor KD, Fleming CR, Abrams A, Hirschkorn MA. Liver function values in adults receiving total parenteral nutrition. JAMA 1979; 241: 2398-400.

50. Bower RH. Hepatic complications of parenteral nutrition. Semin Liv Dis 1983; 3: 216-24.

51. Quigley EMM, Marsh MN, Shaffer JL, Markin RS. Hepatobiliary complications of total parenteral nutrition. Gastroenterology 1993; 104: 286-301.

52. Klein S, Nealon W. Hepatobiliary abnormalities associated with total parenteral nutrition. Sem Liver Dis 1988; 8(3): 237-46.

53. Grant JP, Cox CE, Kleinman LM, Maher MM, Pittman MA, Tangrea JA, *et al.* Serum hepatic enzyme and bilirubin elevations during parenteral nutrition. Surg Gynecol Obstet 1977; 145: 573-80.

54. Sheldon GF, Peterson SR, Sanders R. Hepatic dysfunction during hyperalimentation. Arch Surg 1978; 113: 504-8.

55. Host WR, Serlin O, Ruxh BF. Hyperalimentation in cirrhotic patients. Am J Surg 1972; 123: 57-62.

56. Lowry SF, Brennan MF. Abnormal liver function during parenteral nutrition: Relation to infusion excess. J Surg Res 1979; 26: 300-7.

57. Allardyce DB, Salvian AJ, Quenvelle NF. Cholestatic jaundice during total parenteral nutrition. Can J Surg 1978; 21: 332-9.

58. Tulikoura I, Huikuri K. Morphological fatty changes and function of the liver, serum free fatty acids, and triglycerides during parenteral nutrition. Scand J Gastroenterol 1982; 17: 177-85.

59. Kaminski DL, Adams A, Jellinek M. The effect of hyperalimentation on hepatic lipid content and lipogenic enzyme activity in rats and man. Surgery 1980; 88: 93-100.

60. Hall RI, Grant JP, Ross LH, Coleman RA, Bozovic MG, Quarfordt SH. Pathogenesis of hepatic steatosis in the parenterally fed rat. J Clin Invest 1984; 74: 1658-68.

61. Meguid MM, Akahoshi MP, Jeffers S, *et al.* Amelioration of metabolic complications of conventional total parenteral nutrition. Arch Surg 1984; 119: 1294-8.

62. Allardyce DB. Cholestasis caused by lipid emulsions. Surg Gynecol Obstet 1982; 154: 641-7.

63. Bowyer BA, Fleming CR, Ludwig J, Petz J, McGill DB. Does long-term home parenteral nutrition in adult patients cause chronic liver disease? J Parenter Enter Nutr 1985; 9: 11-7.

64. Stanko RT, Nathan G, Mendelow H, Adibi S. Development of hepatic cholestasis and fibrosis in patients with massive loss of intestine supported by prolonged parenteral nutrition. Gastroenterology 1987; 92(1): 197-202.

65. Hodes JE, Grosfeld JL, Weber TR, Schreiner RL, Fitzgerald JF, Mirkin LD. Hepatic failure in infants on total parenteral nutrition (TPN): Clinical and histopathologic observations. J Ped Surg 1982; 17(5): 463-8.

66. Brown MR, Thunberg BJ, Golub L, Namiscalo WM, Cox C, Shapiro DL. Decreased cholestasis with enteral instead of intravenous protein inthe very low-birth weight infant. J Pediatr Gastroenterol Nutr 1989; 9: 21-7.

67. Cohen C, Olsen MM. Pediatric total parenteral nutrition. Liver histopathology. Arch Pathol Lab Med 1981; 105: 152-6.

68. Vileisis RA, Inwook RJ, Hunt CE. Prospective controlled study of parenteral nutrition-associated cholestatic jaundice: effect of protein intake. J Pediatr 1980; 96: 893-7.

69. Black DD, Suttle EA, Whitington PF, Whitington GL, Korones DS. The effect of short-term total parenteral nutrition on hepatic function in the human neonate: a prospective randomized study demonstrating alteration of hepatic canalicular function. J Pediatr 1981; 99: 445-9.

70. Belli DC, Fournier L-A, Lepage G, Yousef I, Weber AM, Tuchweber B, Roy CC. Total parenteral nutrition-associated cholestasis in rats: comparison of different amino acid mixtures. J Parenter Enter Nutr 1987; 11: 67-73.

71. Belli DC, Roy CC, Fournier L-A, Tuchweber B, Giguere R, Yousef IM. The effect of taurine on the cholestatic potential of sulfated lithocholate and its conjugates. Liver 1991; 11: 162-9.

72. Guertin F, Roy CC, Lepage G, Perea A, Giguere R, Yousef I, Tuchweber B. Effect of taurine on total parenteral nutrition-associated cholestasis. J Parenter Enter Nutr 1991; 15: 247-51.

73. Hancock BJ, Wiseman NE. Lethal short-bowel syndrome. J Ped Surg 1990; 25(11): 1131-4.

74. McIntyre PB, Fitchew M, Lennard-Jones JE. Patients with a high jejunostomy do not need a special diet. Gastroenterology 1986; 91: 25-33.

75. Woolf GM, Miller C, Kurian R, Jeejeebhoy KN. Nutritional absorption in short bowel syndrome: Evaluation of fluid, calorie, and divalent cation requirements. Dig Dis Sci 1987; 32(1): 8-15.

76. Thomson JS. Surgical management of short bowel syndrome. Surgery 1993; 113(1): 4-7.

77. Thomson JS. Surgical considerations in the short bowel syndrome. Surg Gynecol Obstet 1993; 176(1): 89-101.

78. Warner BW, Chaet MS. Nontransplant options for management of the short bowel syndrome. J Ped Gastoenterol and Nutr 1993; 17(1): 1-12.

79. Kurz R, Sauer H. Treatment and metabolic finding in extreme short bowel syndrome with 11 cm jejunal remnant. J Ped Surg 1983; 18: 257-63.

80. Warden MJ, Wesley JR. Small bowel reversal procedure for treatment of the short gut baby. J Ped Surg 1978; 13: 321-3.

81. Wilmore DW, Johnson DJ. Metabolic effects of small bowel reversal in treatment of the short bowel syndrome. Arch Surg 1968; 97: 784-91.

82. Garcia VF, Templeton JM, Eichelberger MR, *et al*. Colon interposition for the short bowel syndrome. J Ped Surg 1981; 16. 994-5.

83. Glick PL, de Lorimier AA, Adzick NS, Harrison MR. Colon interposition: An adjuvant operation for short gut syndrome. J Ped Surg 1984; 19: 719-25.

84. Stahlgren LH, Roy RH, Umana G. A mechanical impediment to intestinal flow: Physiological effects on intestinal absorption. JAMA 1964; 187: 41-4.

85. Ricotta J, Zuidema GD, Gadacz TR, Sadri D. Construction of an ileocecal valve and its role in massive resection of the small intestine. Surg Gynecol Obstet 1981; 152: 310-4.

86. Gareskey J, Weber TR, Grosfeld JL. Ileocecal valve replacement. Its effect on transit time, survival, and weight change after massive intestinal resection. Arch Surg 1981; 116: 618-22.

87. Waddell WR, Kern F, Halgrimson CG, Woodbury JJ. A simple jejunocolic "valve" for relief of rapid transit and the short bowel syndrome. Arch Surg 1970; 100: 438-44.

88. Vinograd I, Merguerian P, Udassin R, Mogle P, Nissan S. An experimental model of a submucosally tunneled valve for the replacement of the ileo-cecal valve. J Ped Surg 1984; 19: 726-31.

89. Hidalgo F, Lopez Cortes M, Salas SJ, Zavala J. Intestinal muscular layer ablation in short bowel syndrome. Arch Surg 1973; 106: 188-90.

90. Weber TR, Vane DW, Grosfeld JL. Tapering enteroplasty in infants with bowel atresia and short gut. Arch Surg 1982; 117: 684-8.

91. Ramanujan TM. Functional capability of blind small loops after intestinal remodeling techniques. Aust N Z J Surg 1986; 54: 145-50.

92. Bianchi A. Intestinal loop lengthening -- A technique for increasing small intestinal length. J Ped Surg 1980; 15: 145-51.

93. Boeckman CR, Traylor R. Bowel lengthening for short gut syndrome. J Ped Surg 1981; 16: 996-7.

94. Bianchi A. Intestinal lengthening: An experimental and clinical review. J R Soc Med 1984; 77: 35-41.

95. Thomson JS, Pinch LW, Murray N, Vanderhoof JA, Schultz LR. Intestinal experience with lengthening for the short bowel syndrome. J Ped Surg 1991; 26: 721-4.

96. Vanderhoof JA, Thompson JS, Murray ND, Kaufman SS, Pinch LW. Gut lengthening (Bianchi) procedure in short bowel syndrome. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O27.

97. Cezard JP, Aigrain Y, Peuchmaur M, Bellaiche M, Ferkdadji L, Ovetchkine P, *et al.* Longitudinal division of small intestine in five children. An alternative to small bowel transplantation. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O29.

98. Huskisson LJ, Brereton RJ, Kiely EM, Spitz L. Problems with intestinal lengthening. J Ped Surg 1993; 28(5): 720-2.

99. Ienaga T, Kimura K, Hashimoto K, Lee SC, Brakstad M, Soper RT. Isolated bowel segment (Iowa model I): Technique and histological studies. J Ped Surg 1990; 25: 902-4.

100. Yamazato M, Kimura K, Yoshino H, Soper RT. The isolated bowel segment (Iowa model II) created in functioning bowel. J Ped Surg 1991; 26: 780-3.

101. Yoshino H, Kimura K, Yamazata M, Scott DH, Soper RT. The isolated bowel segment (Iowa model II): Absorption studies for glucose and leucine. J Ped Surg 1991; 26: 1372-5.

102. Yamazato M, Kimura K, Yoshino H, Murr M, Ellsburg D, Soper RT. The isolated bowel segment (Iowa model II): Motility across the anastomosis with or without mesenteric division. J Ped Surg 1992; 27: 691-5.

103. Kimura K, Spencer RT. A new bowel elongation technique for the short-bowel syndrome using the isolated bowel segment Iowa models. J Ped Surg 1993; 26(6): 792-4.

104. Thomson JS, Vanderhoof JA, Antonson DL, Newland JR, Hodgson PE. Comparison of techniques for growing small bowel neomucosa. J Surg Res 1984; 36: 401-6.

105. Thomson JS, Kampfe PW, J.R. Newland JR & J.A. Vanderhoof, Growth of intestinal neomucosa on prosthetic materials, J Surg Res, 41, 484-92, 1986.

106. Lillemoe KD, Berry WR, Harmon JW Tai YH, Weichbrod RH, Cogen MA. Use of vascularized abdominal wall pedicle flaps to grow small bowel neomucosa. Surgery 1982; 91: 292-300.

107. Watson LE, Friedman HI, Griffin DG, Norton LW, Mellick PW. Small bowel neomucosa. J Surg Res 1980; 28: 280-91.)

108. Barlas M, Gokcora H, Erekul S, Dindar H, Yucesan S. Human amniotic membrane as an intestinal patch for neomucosal growth in the rabbit model. J Ped Surg 1992; 27(5): 597-601.

109. Binnington HB, Tumbleson ME, Ternberg JL. Use of jejunal neomucosa in the treatment of short gut syndrome in pigs. J Ped Surg 1975; 10: 617-21.

110. Thomson JS, Harty RJ, Saigh JA, Giger DK. Morphological and nutritional responses to intestinal patching following intestinal resection. Surgery 1988; 103: 79-86.

111. Ingham-Clark CL, Lear PA, Wood S, Lennard-Jones JE, Wood RFM. Potential candidates for small bowel transplantation. Br J Surg 1992; 79: 676-9.

112. Talsma SE, Marks WM, Marks C, Brailz M. Potential recipients for small bowel transplantation in the United States and the United Kingdom. In; Deltz E, Thicke A, Hamelmann H, editors (eds), Small Bowel Transplantation: Experimental and Clinical 'Fundamentals, Berlin, Springer, 258-61, 1986.

113. Wallander J, Ewald U, Lackgren G, Tufveson G, Wahlberg J, Meurling S. Extreme short bowel syndrome in neonates: An indication for small bowel transplantation? Transplant Proc 1992; 24(3): 1230-5.

114. Tzakis AG, Todo S, Reyes J, *et al.* Clinical intestinal transplantation: Focus on complications. Transplant Proc 1992; 24: 1238-40.

115. Alican F, Hardy JD, Cayir'i M, Varner JE, Moynihan PC, Turner MD, Anas P. Intestinal transplantation: Laboratory experience and report of a clinical case. Am J Surg 1971; 121: 150-9.

116. Revillon Y, Jan D, Sarnacki S, Goulet O, Brousse N, Ricour C. Intestinal transplantation in the child: Experimental and clinical studies. J Ped Surg 1993; 28(3): 292-8.

117. Reznick RK, Craddock GN, Langer B, Gilas T, Cullen JB. Structure and function of small bowel allografts in the dog: Immunosuppression with Cyclosporin A. Can J Surg 1982; 25(1): 51-5.

118. Carrel A. La technique operatoire des anastomoses vasculaires et la transplantation des visceres. Lyon MFO 1902; 98: 859-64.

119. Lillehei R, Idezuki Y, Feemster JA, Dietzman RH, Kelly WD, Goetz FC, *et al.* Transplantation of stomach, intestine and pancreas: Experimental and clinical observations. Surgery 1967; 62(4): 721-41.

120. Stauffer UG. Der gegenwartige stand der dunndarmstransplantation. Padiatr Padol (Suppl) 1975; 3: 59.

121. Ru[illegible], Lillehei RL. Intestinal transplantation. Am J Proctol 1972; 23: 379-93.

122. Fortner JG, Sichuk G, Litwin S, Beattie EJ, Jr. Immunological responses to an intestinal allograft with HL-A-identical donor-recipient. Transplantation 1972; 14(5): 531-5.

123. Goulet O, Revillon Y, Jan D, Brousse N, De Potter S, Cerf-Bensussan N, *et al.* Small-bowel transplantation in children. Transplant Proc 1990; 22(6): 2499-500.

124. Goulet O, Revillon Y, Brousse N, Jan D, Canion D, Rambaud C, *et al.* Successful small bowel transplantation in an infant. Transplantation 1992; 53(4): 940-3.

125. Goulet O, Revillon Y, Brousse N, Colomb V, Jan D, Caniomi D, *et al.* Normal growth four and half years after small bowel transplantation using Cyclosporine A in an infant. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O7.

126. Grant D, Wall W, Mimeault R, Zhong R, Ghent C, Garcia B, *et al.* Successful small-bowel-liver transplantation. Lancet 1990; 335: 181-4.

127.. Grant D, Wall W, Ghent C, Atkinson P, Williams S, Garcia B, *et al.* Small bowel transplantation at University ospital, London, Ontario, Canada. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O3.

128. Todo S, Tzakis A, Reyes J, Abu-Elmagd K, Casavilla A, Nour BM, *et al.* Clinical small bowel or small bowel plus liver transplantation under FK506. Transplant Proc 1991; 23(6): 3093-5.

129. Todo S, Tzakis AG, Abu-Elmagd K, Reyes J, Fung JJ, Casavilla A, *et al.* Cadaveric small bowel and small bowel-liver transplantation in humans. Transplantation 1992; 53(2): 369-76.

130. Tzakis AG, Fung JJ, Todo S, Reyes J, Green M, Starzl TE. Use of FK506 in pediatric patients. Transplant Proc 1992; 23(1): 924-7.

131. Todo S, Tzakis A, Abu-Elmagd K, Reyes J, Furukawa H, Nour B, *et al.* Clinical intestinal transplantation Transplant Proc 1993; 25(3): 2195-7.

132. Todo S, Tzakis A, Reyes J, Abu-Elmagd K, Casavilla A, Fung JJ, Starzl TE. Intestinal transplantation in humans under FK506. Transplant Proc 1993; 25(1): 1198-9.

133. Todo S, Tzakis A, Reyes J, Abu-Elmagd K, Furukawa H, Nour B, *et al.* Clinical intestinal transplantation: three-year experience. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O2.

134. Dearden D, Pollard S, Bianchi A, Calne R. The Manchester and Cambridge (UK) experience of clinical small bowel transplantation. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O4.

135. Langnas AN, Vanderhoof JA, Antonson DL, Mack DR, Fox IJ, Heffron TG, *et al.* Combined liver/small intestinal transplantation in children. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O6.

136. Foster PF, Sankary HN, McChesney L, Xiao F, Kociss K, Koukoulis G, Williams JW. Splanchnic transplantation. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O5.

137. Luck R, Klempnauer J, Steiniger B. Simultaneous occurrence of graft-versus-host and host-versus-graft reactions after allogenic MHC class II disparate small bowel transplantation in immunocompetent rats. Transplant Proc 1991; 23(1): 677-8.

138. Halloran PF, Cockfield SM, Madrenen J. The molecular immunology of transplantation and graft rejection. Immunol and Allergy Clin of N Amer 1989; 9: 1-19.

139. Auchincloss H, Jr., Sachs DH. Transplantation and graft rejection. In: Paul WE, editor. Fundamental immunology. New York: Raven Press, 1993: 1099-41.

140. Hanto DW, Mohanakumar T. Transplantation and immunology: Transplant immunology. In: Greenfield LJ, Mulholland MW, Oldham KT, Zelenock GB, editors. Surgery: scientific principles and practices. Philadelphia: J.B. Lippincott Company, 1993: 461-550.

141. Kahan BD. Immunosuppressive therapy. Current Opin Immunol 1992; 4: 553-60.

142. Fischer A. Immune Tolerance and Graft [Lecture]. The IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris.

143. Springer TA. Adhesion receptors of the immune system. Nature 1990; 346: 425.

144. Monchik GJ, Russell PS. Transplantation of small bowel in the rat: Technical and immunological considerations. Surgery 1971; 70(5): 693-702.

145. Iwaki Y, Starzl TE, Yagihashi A, Taniwaki S, Abu-Elmagd K, Tzakis A, *et al.* Replacement of donor lymphoid tissue in small-bowel transplants. Lancet 1991; 337: 818-9.

146. Ingham-Clark CL, Cunningham AJ, Crane PW, Wood RFM, Lear PA. Lymphocyte infiltration patterns in rat small-bowel transplants. Transplant Proc 1990; 22(6): 2460.

147. Rosenurgy AS, Schraut WH. Small bowel allografts: Sequence of histological changes in acute and chronic rejection. Am J Surg 1986; 151: 470-5.

148. Schmid T, Oberhuber G, Korozsi G, Klima G, Margreiter R. Histologic pattern of small bowel allograft rejection in the rat: Mucosal biopsies do not provide sufficient information. Gastroenterology 1989; 96: 1529-32.

149. Kirkman RL, Lear PA, Madara L, Tilney NL. Small intestine transplantation in the rat -- immunology and function. Surgery 1984; 96(2): 280-6.

150. Crane PW, Lear PA, Sowter C, Davies RL, Slavin G, Wood RFM. Early microvascular changes in small intestinal allografts. Transplant Proc 1989; 21(1): 2906.

151. Schraut WH, Lee KKW, Dawson PJ, Hurst RD. Graft-versus host disease induced by small bowel allografts: Clinical course and pathology. Transplantation 1986; 41(3): 286-90.

152. Grant D, Zhong R, Gunn H, Stiller C, Garcia B, Keown P, Duff J. Graft-versus-host reactions induced by intestinal transplantation in the rat produce host immunosuppression. Transplant Proc 1988; 20(6): 1279-80.

153. Deltz E, Ulrichs K, Schack, Friedrichs TB, Muller-Ruchholtz W, Muller-Hermelink HK, Thiede A. Graft-versus-host reaction in small bowel transplantation and possibilities for its circumvention. Am J Surg 1986; 151:1 379-86.

154. Piernne J, Lardinois R, D'Silva M, Fridman V, Bonivera J, Mahieu P, *et al.* Relevance of mesenteric lymph nodes to graft-versus-host disease following small bowel transplantation. Transplantation 1990; 50: 711-4.

155. Wallander J, Lackgren G, Tufveson G. T-lymphocytes are necessary for fatal graft vs host disease after small bowel transplantation. Transplant Proc 1989; 21(1): 2896-7.

156. Ingham Clark CL, Price BA, Crane PW, Lear PA, Wood RFM. Persistence of allogeneic cells in graft and host tissues after small bowel transplantation. Br J Surg 1992; 79: 424-6.

157. Kobayashi E, Kamada N, Enosawa S, Toyama N, Delriviere L, Goto S, *et al.* Comparison of potentiality to induce graft-versus-host reaction with small bowel, pancreas/spleen, and liver transplantation in the rat. Clin Exp Immunol 1993; 92: 527-31.

158. Tanabe M, Murase N, Demetris AJ, Nakamura K, Furuya T, Fujisaki S, *et al.* Graft-versus-host disease in fully allogeneic small bowel transplantation: Incidence of the disease and strain combinations. Transplant Proc 1993; 25(1): 1214-5.

159. Diflo T, Maki T, Balogh K, & A.P. Monaco AP. Graft-versus-host disease in fully allogeneic bowel transplantation in the rat. Transplantation 1989; 47(1): 7-11.

160. Fujuwara H, Raju S, Grogan JB, Lewin JR, Johnson WW. Total orthotopic small bowel allotransplantation the dog: Features of an atypical rejection and graft-versus-host reaction. Transplantation 1987; 44: 747-53.

161. Cohen Z, MacGregor AB, Moore KTH, Falk RE, Langer B, Cullen JB. Canine small bowel transplantation: A study of the immunological responses. Arch Surg 1976; 111: 248-53.

162. Kizilisik AT: GVHD in a rat small bowel transplantation model. [M.Sc. thesis]. Edmonton (AB): University of Alberta, 1994.

163. Monaco AP. [comments]. The IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris.

164. Jacques P, D'Silva M, Jacquet N. Survival advantage by transplanting small bowel with donor derived lymphoid tissue. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O88.

165. Starzl TE, Rowe MI, Todo S, Jaffe R, Tzakis A, Hoffman AL, *et al.* Transplantation of multiple abdominal viscera. JAMA 1989; 261(10): 1449-57.

166. Williams JW, Sankary HN, Foster PF, Lowe J, Goldman HM. Splanchnic transplantation: An approach to the infant dependent of parenteral nutrition who develops irreversible liver disease. JAMA 1989; 261(10): 1458-62.

167. Roitt I, Brostoff J, Male D. Immunology. St. Louis: C.V. Mosby Company, 1985.

168. Teitelbaum DH, Narasimhan A, Chenault RH, Merion RM Lymphocyte immunologic interactions in intestinal transplantation. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O76.

169. Lee KKW, Schraut WH. In vitro allograft irradiation prevents graft-versus-host disease in small-bowel transplantation. J Surg Res 1985; 38(4): 364-72.

170. Hasuike Y, Shaked A, Monden M, Shirashi M, Farmer D, Holly EG, Busuttil RW. Selective mesenteric lymphoid irradiation eliminates the effector cells of lethal graft-versus-host disease while protecting radiosensitive intestinal tissue. Transplant Proc 1993; 25(1): 1196-7.

171. Stangl MJ, Langrehr J, Lee KKW, Moynihan HL, Banner B, Schraut WH. Immunologic and metabolic consequences of in vitro and in vivo irradiation of small bowel allografts. Transplant Proc 1990; 22(4): 2058-9.

172. Saat RE, De Bruin RWF, Heineman E, Jeekel J, Marquet RL. Irradiation of the donor combined with Cyclosporine A treatment of the recipient prevents graft-versus-host disease and leads to prolonged survival of small-bowel allografts. Transplant Proc 1990; 22(6): 2465-6.

173. Grant D, Duff J, Zhong R, Mimeault R, Inch R, Stiller C. Effect of ex vivo allograft irradiation combined with Cyclosporine therapy in a pig intestinal transplant model. Transplant Proc 1989; 21(1): 2879-80.

174. Donaldson SS. Nutritional consequences of radiotherapy. Cancer Res 1977; 37: 2407-13.

175. Schaffer D, Maki T, DeMichele SJ, Karlstad MD, Bistrian BR, Balogh K, Monaco AP. Studies in small bowel transplantation: Prevention of graft-versus-host disease with preservation of allograft function by donor pretreatment with antilymphocyte serum. Transplantation 1988; 45(2): 262-9.

176. Diflo T, Maki T, Monaco AP. The existence of graft-versus-host disease in long-term surviving fully allogeneic rat small bowel transplant recipients. Transplant Proc 1989; 21(1): 2875-6.

177. DeBruin RWF, Saat RE, Heineman E, Jeekel J, Marquet RL. Effects of donor pretreatment with antilymphocyte serum and cyclosporin on rejection and graft-versus-host disease after small bowel transplantation in immunosuppressed and nonimmunosuppressed rats. Transpl Int 1993; 6: 22-5.

178. Schaffer D, Simpson MA, Milford EL, Gottschalk R, Kut JP, Maki T, Monaco AP. Donor pretreatment with monoclonal antibody for prevention of graft-versus-host disease following small bowel transplantation: Effect of depletion of T-cell subsets. Transplant Proc 1991; 23(1): 679-81.

179. Li X, Tucker J, Zhong R,. Jevnikar A, Grant D. The role of gut-associated lymphoid tissue in intestinal rejection: Allogeneic response to rat intestinal lymphocytes. Transplantation 1993; 56(1): 244-7.

180. Murase N, Demetris AJ, Kim DG, Todo S, Fung JJ, Starzl TE. Rejection of multivisceral allografts in rats: A sequential analysis with comparison to isolated orthotopic small-bowel and liver grafts. Surgery 1990; 108: 880-9.

181. Sabin S. Mechanism of rejection: Small bowel specificity [lecture]. From: The IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris.

182. Kimura K, Money SR, Jaffe BM. The effects of size and site of origin of intestinal grafts on small-bowel transplantation in the ra. Surgery 1987; 101(5): 618-22.

183. Kimura K, Money SR, Jaffe BM. Short-segment orthotopic intestinal isografts and allografts in enterectomized rats. Transplantation 1987; 44(4): 579-82.

184. Hanson WR, Osborne JW, Sharp JG. Compensation by the residual intestine after intestinal resection in the rat. Gastroenterology 1977; 72: 692-700.

185. Kimura K, LaRosa CA, Money SR, Jaffe BM. Segmental intestinal transplantationin rats with resected entire small bowel, ileocecal valve and cecum. J Surg Res 1988; 45: 349-56.

186. Oki K,Maeda K, Nakamura K. Orthotopic small intestine transplantation in the rat--How long a small intestinal graft is necessary? Transplant Proc 1989; 21(1): 2909-12.

187. Taguchi T, Hirata Y, Hirose R, Yamada T, Suita S. Short segmental in' ransplantation in rats -- comparison of jejunal and ileal grafts. Transplant Proc 1992; 24(3). 1079-80.

188. Kimura K, Larosa CA, Blank MA, Jaffe BM. Successful segmental intestinal transplantation in enterectomized pigs. Ann Surg 1990; 211(2): 158-64.

189. Benchimol D, Pesce A, Delque-Bayer P, Saint-Paul MC, Giudicelli J, Mouroux J, *et al.* Jejunal versus ileal segmental allografts in the dog: Comparison of immunologic and functional results. Surgery 1992; 112(5): 918-27.

190. Kimura K, Money SR, Jaffe BM. The effects of Cyclosporin on varying segments of small-bowel grafts in the rat. Surgery 1988; 104(1): 64-9.

191. Meijssen MAC, Heineman E, DeBruin RWF, Wolvekamp CJ, Marquet RL, Molenaar JC. Long-term survival of DLA-matched segmental small-bowel allografts in dogs. Transplantation 1993; 56(5): 1062-6.

192. Kahan BD. Transplantation timeline: Mankind's three millennia - one maverick's three decades in the struggle against biochemical individuality. Transplantation 1991; 51(1): 1-13.

193. Cohen DJ, Loertscher R, M. Rubin M, Tilney NL, Carpenter CB, Strom TB. Cyclosporin : A new immunosuppressive agent for organ transplantation. Ann Int Med 1984; 101: 667-82.

194. Morris PJ. Cyclosporin A. Transplantation 1981; 32(5): 349-54.

195. Kahan BD. Cyclosporin. New Engl J Med 1989; 321(25): 1725-38.

196. Schreiber SL, Crabtree GR. The mechanism of action of CyA and FK506. Immunol Today 1992; 13(4): 136-42.

197. Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-CyA and FKBP-FK506 complexes. Cell 1991; 66: 807-15.

198. Flanagan WM, Corthesy B, Bram RJ, Crabtree GR. Nuclear association of a T-cell transcription factor blocked by FK506 and cyclosporin A. Nature 1991; 352: 803-7.

199. Kunz J, Hall MN. Cyclosporin A, FK506 and rapamycin: more than just immunosuppression. TIBS 1993; 18: 334-8.

200. McCaffrey PG, Perrino BA, Soderling TR and Rao A. NF-AT$_p$, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. J Biol Chem 1993; 268(5): 3747-52.

201. McCaffrey PG, Luo C, Kerppola TK, Jain J, Badalian TM, Ho AM, *et al.* Isolation of the cyclosporin-sensitive T cell transcription factor NFAT$_p$. Science 1993; 262: 750-4.

202. Zipfel PF, Irving SG, Kelly K, Siebenlist V. Complexity of the primary genetic response to mitogenic activation of human T-cells. Mol Cell Biol 1989; 9(3): 1041-8.

203. Johanson A, Moller E. Evidence that the immunosuppressive effect of FK506 and cyclosporin are identical. Transplantation 1990; 50; 1001-7.

204. Craddock GN, Nordgren SR, Reznick RK, Gilas T, Lossing AG, Cohen Z, *et al.* Small bowel transplantation in the dog using Cyclosporine. Transplantation 1983; 35(4): 284-8.

205. Lee KK, Hurst RD, Schraut WH. Enteral Cyclosporin A absorption following orthotopic small-bowel transplantation. Gastroenterology 1987; 92: 1497.

206. American Association for Clinical Chemistry Task Force on Cyclosporin Monitoring. Critical issues in Cyclosporin monitoring: Report of the task force on Cyclosporin monitoring. Clin Chem 1987; 33(7): 1269-88.

207. Grant D, Duff J, Zhong R, Garcia B, Lipohar C, Keown P, Stiller C. Successful intestinal transplantation in pigs treated with cyclosporin. Transplantation 1988; 45(2): 279-84.

208. Harmel RP, Jr., Stanley M. Improved survival after allogeneic small intestinal transplantation in the rat using Cyclosporine immunosuppression. J Ped Surg 1986; 21(3): 214-7.

209. Lee KKW, Schraut WH. Structure and function of orthotopic small bowel allografts in rats treated with Cyclosporine. Am J Surg 1986; 151: 55-60.

210. Schraut WH, Lee KKW, Sitrin M. Recipient growth and nutritional status following transplantation of segmental small-bowel allografts. J Surg Res 1987; 43: 1-9.

211. Shimazu R, Grogan JB, Raju S. Long-term survival of orthotopic bowel allografts in the rat treated with short-term low-dose Cyclosporine. Transplantation 1988; 46(5): 673-7.

212. Sigalet DL, Kneteman NM, ThomsonABR. Cyclosporin reduces nutrient absorption by normal bowel. Clin Res 1991; 39: 18A.

213. Bruserud O, Pawelec G. Cyclosporine A and FK506 show similar immunosuppressive effects on long-term in vitro T-cell proliferation. Int J Immunopharmac 1993; 15(2): 93-7.

214. Harmel RP, Jr., Tutschka PJ, Sonnino RE, Teitelbaum DH. Immune tolerance of intestinal allografts in the rat. Transplant Proc 1989; 21(1): 2883-4.

215. Cohen Z, Silverman R, Wassef R, Levy GA, Burnstein M, Cullen J, et al. Small intestinal transplantation using Cyclosporine: Report of a case. Transplantation 1986; 42(6): 613-21.

216. Cohen Z, Silverman R, Levy G, Wassef R, Langer B. Clinical small intestinal transplantation using Cyclosporine A and Methylprednisolone. Transplant Proc 1987; 19: 2588-90.

217. Revillon Y. [Introduction to the symposium]. The IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris.

218. Murray BM, Paller MS, Ferris TF. Effect of Cyclosporine administration on renal hemodynamics in conscious rats. Kidney Int 1985; 28: 767-74.

219. Kahan BD. Cyclosporin. N Engl J Med 1989; 321: 1725-38.

220. Begnigni A, Morigi M, Percio N, Zoja C, Amuchastegui CS, Piccinelli A, *et al.* The Acute effect of FK506 and Cyclosporine on endothelial cell function and renal vascular resistance. Transplantation 1992; 54(5): 775-80.

221. Textor SC, Weismer R, Wilson DJ, Porayko M, Romero JC, Burnett JC, *et al.* ,Systemic and renal hemosynamic differences between FK506 and Cyclosporine in liver transplant recipients. Transplantation 1993; 55(6): 1332-39.

222. Skorecki KL, Rutledge WP, Schrier RW. Acute cyclosporin nephrotoxicity-Prototye for a renal membrane signalling disorder. Kidney Internat 1992; 42: 1-10.

223. Gillum DM, Truong L, Tasby J, Migliore P, Suki WN. Chronic Cyclosporine nephrotoxicity. A rodent model. Transplantation 1988; 46: 285-92.

224. Olbricht CJ, Grone HJ, Bossaller C, Gutjahr E, Hertel RF. Alterations in proteolytic enzymes of the proximal tubule in a rat model of Cyclosporine nephrotoxicity. Transplantation 1990; 50(3): 378-81.

225. June CH, Thompson CB, Kennedy MS, Nims J, Thomas ED. Profound hypomagnesemia and renal magnesium wasting associated with the use of cyclosporine for marrow transplantation. Transplantation 1985; 39: 620-4.

226. Marsh JW, Vehe KL, White HM. Immunosuppressants. astoent clin of N Amer 1992; 21(3): 679-93.

227. Starzl TE, Abu-Elmagd K, Tzakis A, Fung JJ, Porter KA, Todo S. Selected topics on FK506, with special references fo rescue of extrahepatic whole organ grafts, transplantation of "forbidden organs", side effects, mechanisms, and practical pharmacokinetics. Transplant Proc 1991; 23(1): 914-9.

228. de Groen PC, Aksamit AJ, Rakela J, Forbes GS, Krom RAF. Central nervous system toxicity after liver transplantation: the role of cyclosorin and cholesterol. N Engl J Med 1987; 317: 861-6.

229. Kahan BD, Flechner SM, Lorber MI, Golden D, Conley S, Van Buren CT. Complications of cyclosporin-prednisone immunosuppression in 402 renal alograft recipients exclusively followed at a single center from one to five years. Transplantation 1987; 43: 197-204.

230. Wysocki GP, Gretzinger HA, Laupacis A, Ulan RA, Stiller CR. Fibrous hyperplasiz of the gingiva: A side effect of cyclosporin A therapy. Oral Surg 1983; 55(3): 274-8.

231. Krentz AJ, Dousset B, Mayer D, McMaster P, Buckles J, Cramb R, *et al.* Metabolic effects of cyclosporin A and FK506 in liver transplant recipients. Diabetes 1993; 42: 1753-9.

232. Herold KC, Nagamatsu S, Buse J, Kulsakdinin P, Steiner D. Inhibition of glucose-stimulated insulin release from β-TC3 cells and rodent islets by an analog of FK506. Transplantation 1993; 55(1): 186-92.

233. Mieles L, Gordon RD, Mintz D, Toussaint RM, Imventarza O, Starzl TE. Glycemia and insulin need following FK506 rescue therapy in liver transplant recipients. Transplant Proc 1991; 23(1): 949-53.

234. Dresner LS, Andersen DK, Kahng KU, Munshi IA, Wait RB. Effects of Cyclosporine on glucose metabolism. Surgery 1989; 106: 163-70.

235. Thai BL, Dumont M, Michel A, Erlinger S, Houssin D. Cholestatic effect of Cyclosporine in the rat. Transplantation 1988; 46(4): 510-2.

236. Roman ID, Monte MJ, Gonzalez-Buitrago JM, Estelier A, Jimen R. Inhibition of hepatocytary vesicular transport by Cyclosporin A in the rat: Relationship with cholestasis and hyperbilirubinemia. Hepatology 1990; 12(1): 83-91.

237. Stone B, Warty V, Dindzans V, Van Thiel D, The mechanism of Cyclosporine-induced cholestasis in the rat. Transplant Proc 1988; 20 (suppl 3): 841-4.

238. Stone BG, Udani M, Sanghvi A, Warty V, Plocki K, Bedetti CD, Van Thiel DH. Cyclosporin-A-induced cholestasis: The mechanism in a rat model. Gastroenterol 1987; 93: 344.

239. Bohme M, Buchler M, Muller M, Keppler D. Differential inhibition by cyclosporins of primary-active ATP-dependent transporters in the hepatocyte canalicular membrane. FEBS 1993; 333(1,2): 193.

240. Kadman M, Klunemann C, Bohme M, Ishikawa T, Gorgas K, Otto G, *et al.* Inhibition by cyclosporin A of adenosine triphosphate-dependent transport from the hepatocyte into bile. Gastroenterol 1993; 104: 1507-14.

241. Moseley RH, Johnson TR, Morissette JM. Cyclosporine (CsA) inhibition of taurocholate transport in rat liver plasma membrane vesicles. Gastroenterology 1989; 96: A634.

242. Sigalet DL, Kneteman NM, Fedorak RN, Kizilisik T, Madsen KE, Thomson ABR. Small intestinal function following syngeneic transplantation in the rat. 1993; Submitted.

243. Ballinger WF, Christy, MG, Ashby, WB. Autotransplantation of the small intestine: The effects of denervation. Surgery 1962; 52: 151.

244. Schroeder P, Sandforth F, Deltz E. Glucose absorption after heterotopic small-bowel transplantation. In: Deltz E, editor. Small Bowel Transplantation. Experimental and Clinical Fundamentals. Berlin: Springer, 1986: 74-8.

245. Schroeder P, Deltz E, Seifert J, Sandforth F, Thiede A. Absorptive capacity of the transplanted small bowel. Gut 1987; 28 (suppl 1): 275-9.

246. Watson AJM, Lear PA, Montgomery A, Elliot E, Dacre J, Farthing MJG, Wood RFM. Water, electrolyte, glucose, and glycine absorption in rat small intestinal transplants. Gastroenterology 1988; 94: 863-9.

247. Raju S, Fujiwara H, Grogan JB, Achord JL. Long-term nutritional function of orthotopic small bowel autotransplants. J Surg Res 1989; 46: 112-6.

248. Sigalet DL, Kneteman NM, Fedorak RN, Kizilisik AT, Thomson ABR. Intestinal function following allogeneic small intestinal transplantation in the rat. Transplantation 1992; 53(2): 264-71.

249. Quigley EMM, Thompson JS, Rose SG. The long-term function of canine jejunoileal autotransplants: Insights into allograft physiology. Transplant Proc 1992; 24(3): 1105-6.

250. Ishii H, Hashimoto N, Kitada T, Kuroki T, Utsnomiya J. Malabsorption after small-bowel transplantation. Hepato-Gastroenterol 1993; 40: 282-4.

251. Sigalet DL, Kneteman NM, Thomson ABR. The effects of Cyclosporine on normal bowel. Transplantation 1991: 51(6): 1296-1302.

252. Sigalet DL, Kneteman NM, Thomson ABR. Reduction of nutrient absorption in normal rats by Cyclosporine. Transplantation 1992; 53(5): 1103-7.

253. Collin J, Dennison AR, Watkins RM, Millard PR, Morris PJ. Segmental small intestinal allografts: II. Inadequate function with Cyclosporine immunosuppression: Evidence of a protein-losing enteropathy. Transplantation 1987; 44(4): 479-83.

254. Chan P, Scoble JE, Senior JM, Varghese Z, Sweny P, Moorhead JF. Cyclosporin inhibition of glucose transport in cell culture. Transplant Proc 1989; 21(1): 922-3.

255. Hopfer U. Membrane transport mechanisms for hexoses and amino acids in the small intestine. In: L.R. Johnson LR, editor. Physiology of the Gastrointestinal Tract, 2nd edition. New York: Raven Press, 1987: 1499-1526.

256. Alpers DH. Digestion and absorption of carbohydrates and proteins. In: L.R. Johnson LR, editor. Physiology of the Gastrointestinal Tract, 2nd edition. New York: Raven Press, 1987: 1469-98.

257. Sigalet DL, Kneteman NM, Simpson I, Walker K, Thomson ABR. Intestinal permeability after small intestinal transplantation and Cyclosporine treatment. Transplant Proc 1992; 24(3): 1120-1.

258. Meddings JB. Membrane function: Its relationship to intestinal absorption and malabsorption. Can J Gastroenterol 1990; 4(1): 39-46.

259. Heeckt PF, Halfter WM, Lee KKW, Schraut WH, Bauer AJ. Prolonged in vivo administration of cyclosporine causes enteric smooth muscle hyperplasia in normal rat intestine and small bowel grafts. In Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris. P97.

260. Wallemacq PE, Reding R. FK506 (Tacrolimus), a novel immunosuppressant in organ transplantation: Clinical, biomedical, and analytical aspects. Clin Chem 1993; 39(11): 2219-28.

261. Sadawa S, Suzuki G, Kawase Y, Takaku F. Novel immunosuppressive agent FK506. In vitro effects on the cloned T-cell activation. 1987; 139: 1797-803.

262. Goto T, Kino T, Hatanaka H, Okuhara M, Kohsaka M, Aoki, Imanaka H. FK506. Historical perspectives. Transplant Proc 1991; 23(6): 2713-7.

263. Tocci MJ, Matkovich DA, Collier KA, Kwok P, Dumont R, Lin S, *et al.* The immunosuppressant FK506 selectively inhibits the expression of early T cell activation genes. J Immunol 1989; 143: 718-26.

264. Morikawa K, Oseka F, Morikawa S. The distinct effects of FK506 on the activation , proliferation, and differentiation on human B lymphocytes. Transplantation 1992; 54(6): 1025-30.

265. Muraguchi A, Butler JL, Kehrl JH, Falkoff RJM, Fauci AS. Selective suppression of an early step in human B-cell activation by Cyclosporin A, J Exp Med 1983; 158: 690.

266. Ochiai T, Nagata M, Nakajima K, Sakamoto K, Asano T, & K. Isono K. Prolongation of canine renal allograft survival by treatment with FK506. Transplant Proc 1987; 19 (5 suppl 6): 53-6.

267. Ochiai T, Nakajima K, Nagata M, Suzuki T, Asana T, Uematsu T, *et al.* Effect of a new immunosuppressice agent, FK506, on heterotopic cardiac allotransplantation in the rat. Transplant Proc 1987; 19: 1284-6.

268. Todo S, Podesta L, Chapchap P, Kahn D, Pan CE, Ueda Y, *et al.* Orthotopic liver transplantation in dogs receiving FK506. Transplant Proc 1987; 19(5 suppl 6): 64-7.

269. Hoffman AL, Makowka L, Banner B, Cai X, Cramer DV, Pascualone A, *et al.* The use of FK506 for small intestine allotransplantation: Inhibition of acute rejection and prevention of fatal graft-versus-host disease. Transplantation 1990; 49(3): 483-90.

270. Lee KKW, Stangl MJ, Todo S, Langrehe JM, Starzl TE, SchrautWH. Successful orthotopic small bowel transplantation with short-term FK506 immunosuppressive therapy. Transplant Proc 1990; 22(1)(suppl 1): 78-9.

271. Hatazawa C, Yamaguchi M, Kato T, Koyama K. Effect of FK506 on bowel transplantation in rats. Transplant Proc 1992; 24(3): 1177.

272. Date K, Okajima K, Takeda Y, Isozaki H, Tezuka K, Ryo T. Effect of FK506 on graft survival in rat small intestinal allografts. Transplant Proc 1992; 24(3): 1173.

273. Utsunomiya H, Tanaka K, Uemoto S, Kato H, Nishizawa T, Yamamoto E, Ozawa K. Effect of FK506 on orthotopic small bowel transplantation in rats. Transplant Proc 1992; 24(3): 1191.

274. Matsutani H, Yagi M, Hashimoto T, Iyobe T, Iwasa K, Tomita K, *et al.* Suppression of the histologic changes of GVHD by FK506 in rat small bowel transplantation. Transplant Proc 1992; 24(3): 1185-6.

275. D'Alessandro AM, Kalayoglu M, Pirsch JD, Corwith C, Knechtle SJ, Reed A, Belzer FO. FK506 rescue therapy for resistant rejection episodes in liver transplant recipients. Transplant Proc 1991; 23(6): 2987-8.

276. Lewis WD, Jenkins RL, Burke PA, Winn KM, Schaffer D, Lopez R, Monaco AP. FK506 resue therapy in liver transplant recipients with drug-resistant rejection. Transplant Proc 1991; 23(6): 2989-91.

277. Shaw BW, Jr., Markin R, Stratta R, Langnas A, Donovan J, Sorrell M. FK506 for rescue treatment of acute and chronic rejection in liver allograft recipients. Transplant Proc 1991; 23(6): 2994-5.

278. Gibbs JF, Husberg BS, Klintmalm GB, Backman L, Levy M, McMillan R, *et al.* Outcome analysis of FK506 therapy for acute and chronic rejection. Transplant Proc 1993; 25(1): 622-3.

279. McDiarmid SV, Klintmalm G, Busuttil RW. FK506 rescue therapy in liver transplantation: outcome and complications. Transplant Proc 1991; 23(6): 2996-9.

280. US Multicenter FK506 Liver Study Group. Use of FK506 for the prevention of recurrent allograft rejection after successful conversion from cyclosporine for refractory rejection. Transplant Proc 1993; 25(1): 635-7.

281. Fung J, Abu-Elmagd K, Jain A, Gordon R, Tzakis A, Todo S, *et al.* A randomized trial of primary liver transplantation under immunosuppression with FK506 vs Cyclosporine. Transplant Proc 1991; 23(6): 2977-83.

282. McMillan R, Husberg B, Goldstein R, *et al.* A prospective randomized trial of Cyclosporine vs FK506. In: Abstracts of the XIVth International Congress of the Transplantation Society; 1992 Aug; Paris: P63.

283. Starzl TE, Fung J, Jordan M, Shapiro R, Tzakis A, McCauley J, *et al.* Kidney transplantation under FK506. JAMA 1990; 264(1): 63-7.

284. Japanese FK506 Study Group. Japanese study of FK506 on kidney transplantation: Results of late phase II study. Transplant Proc 1993; 25 (1): 649-54.

285. Randhawa PS, Shapiro R, Jordan M, Starzl TE, Demetris AJ. The histopathological changes associated with allograft rejection and drug toxicity in renal transplant recipients maintained on FK506: Clinical significance and comparison with Cyclosporine. Am J Surg Pathol 1993; 17(1): 60-8.

286. Jordan ML, Shapiro R, Vivas CA, Scantlebury VP, Bhandhawa P, Carrieri G, *et al.* FK506 "rescue" for resistant rejection of renal allografts under primary cyclosporine immunosuppression. Transplantation 1994; 57(6): 860-5.

287. Tzakis AG, Reyes J, Todo S, Nour B, Shapiro R, Jordan M, *et al.* Two-year experience with FK506 in pediatric patients. Transplant Proc 1993; 25(1): 619-21.

288. Griffith BP, Bando K, Hardesty RL, Armitage JM, Keenan RJ, Pham SM. A prospective randomized trial of FK506 versus cyclosporine after human pulmonary transplantation. Transplantation 1994; 57(6): 848-51.

289. Reding R, Wallemacq PE, Lamy ME, Rahier J, Sempoux C, DeBande B, *et al.* Conversion from cyclosporine to FK506 for salvage of immunocompromised pediatric liver allografts. Transplantation 1994; 57(1): 93-100.

290. Rosh JR, Schwersenz AH, Schwartz M, Moran V, Leleiko N, LeLeiko VMN, Miller CM. Therapy with FK506 in pediatric liver recipients. Transplant Proc 1994; 26(1): 152-3.

291. Bumgardner GL, Roberts JP. New immunosuppressive agents. Advance in Liver Transplant 1993; 22(2): 421-49.

292. Eidelman BH, Abu-Elmagd K, Wilson J, Fung JJ, Alessiani M, *et al.* Neurologic complications of FK506. Transplant Proc 1991; 23(6): 3175-8.

293. Alessiani M, Cillo U, Fung JJ, Irish W, Abu-Elmagd K, Jain A, *et al.* Adverse effects of FK506 overdosage after liver transplantation. Transplant Proc 1993; 25(1): 628-34.

294. Iwasaki K, Shiraga T, Nagase K, Hirano K, Nozaki K, Noda K. Pharmacokinetic study of FK506 in the rat. , Transplant Proc 1991; 23(6): 2757-9.

295. McDiarmid SV, Colonna J, Shaked A, *et al.* A comparison of renal function in Cyclosporine and FK506 treated patients after primary orthotopic liver transplantation. In: Abstrsacts of the XIVth International Congress of the Transplantation Society; 1992 Aug; Paris: P65.

296. Ochiai T, Sakamoto K, Gunji Y, Hamaguchi K, Isegawa N, Suzuki T, *et al.* Effects of combination treatment with FK506 and Cyclosporine on survival and vascular changes in renal allograft recipient dogs. Transplantation 1989; 48: 193-7.

297. Fung JJ, Alessiani M, Abu-Elmagd K, Todo S, Shapiro R, Tzakis A, *et al.* Adverse effects associated with the use of FK506. Transplant Proc 1991; 23(6): 3105-8.

298. Backman L, Nicar M, Levy M, Distant D, Eisenstein C, Renard T, *et al.* FK506 trough levels in whole blood and plasma in liver transplant recipients: Correlation with clinical events and side effects. Transplantation 1994; 57(4): 519-25.

299. Shutter LA, Gree JP, Newman NJ, Hooks MA, Gordon RD. Cortical blindness and white matter lesions in a patient receiving FK506 after liver transplantation. Neurology 1993; 43: 2417-8.

300. Tabasco-Minguillan J, Mieles L, Carroll P, Gavaler J, Van Thiel DH, Starzl TE. Long-term insulin requirements after liver transplantation with FK506 in American veterans. Transplant Proc 1993; 25(1): 677-8.

301. Ueki M, Yasunami Y, Ina K, Ryu S, Funakoshi A, Kamei T, Ikeda S. Diabetogenic effects of FK506 on renal subcapsular islet isografts in rat. Diabetes Res Clin Pract 1993; 20(1): 11-19.

302. Jindal RM, Popescue I, Emre S, Schwartz ME, Boccagni P, Meneses P. Serum lipid changes in liver transplant recipients in a prospective trial of cyclosporine versus FK506. Transplantation 1994; 57(9): 1395-7.

303. Mor E, Schwersenz A, Sheiner PA, Emre S, Schwartz ME, Miller CM. Reversal of gastrointestinal toxicity assiciated with long-term FK506 immunosuppression by conversion to cyclosporine in liver transplant recipients. Transplantation 1994; 57(7): 1130-1.

304. Sehgal SN, Baker H, Vezina C. Rapamycin (A4-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. J. Antibiot 1975; 28: 727-32.

305. Martel RR, Klicius J, Galet S. Inhibition of the immune response by rapamycin, a new antifungal antibiotic. Can J. Physiol Pharmacol 1977; 55: 48-51.

306. Dumont FJ, Melino MR, Staruch MJ, Koprak SL, Fischer PA, Sigal NH. The immunosuppressive macrolides FK506 and rapamycin act as reciprocal antagonists in murine T-cells. J. Immunol 1990; 144: 1418-24.

307. Bierer BE, Marrila PS, Standaert RF, Herzenberg LA, Burakoff SJ, Crabtree, Schreiber SL. Two distinct signal tranmission pathways in T-lymphocyes are inhibited by complexes formed between an immunophilin and either FK506 or Rapamycin. Proc Natl Acad Sci. USA 1990; 87: 9231-5.

308. Dumont FJ, Staruch MJ, Koprak SL, Melino MR, Sigal NH. Distinct mechanism of suppression of murine T-cell activation by the related macrolides FK506 and rapamycin. J. Immunol 1990; 144(1): 251-8.

309. Henderson DJ, Naya I, Bundick RV, Smith GM, Schmidt JA. Comparison of the effects of FK506, cyclosporin A and rapamycin on interleukin-2 production. Immunol 1991; 73: 316-21.

310. Sigal NH, Lin CS, Siekierka JJ. Inhibition of human T-cell activation by FK506, Rapamycin and Cyclosporin A. Transplant Proc 1991; 23(2) (suppl 2): 1-5.

311. Hamashima T, Yoshimura N, Ohsaka Y, Oka T, Stepkowski S, Kahan B. In vivo use of rapamycin suppresses neither IL-2 production nor IL-2 receptor expression in rat transplant model. Transplant Proc 1993; 25(1): 723-4.

312. Morice WG, Brunn GJ, Wiederrecht G, Siekierka JJ, Abraham RT. Rapamycin-induced inhibition of $p34^{cdc2}$ kinase activation is associated with G_1/S-phase growth arrest in T-lymphocytes. J. Biol Chem 1993; 268(5): 3734-8.

313. Jayaraman T, Marks AR. Rapamycin-FKBP12 blocks proliferation, induces differentiation, and inhibits cdc2 kinase activity in a myogenic cell line. J Biol Chem 1993; 268(34): 25385-8.

314. Kuo CJ, Chung J, Fiorentino DF, Flanagan WM, Blenis J, Crabtree GR. Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. Nature 1992; 358: 70-3.

315. Tsai M, Chen R-H, Tam S-Y, Blenis J, Galli SJ. Activation of MAP kinases, pp90^{rsk} and pp70-S6 kinases in mouse mast cells by signaling through the c-*kit* receptor tyrosine kinase of $Fc_{\varepsilon}RI$: Rapamycin inhibits activation of pp70-S6 kinase and proliferation in mouse mast cells. Eur J Immunol 1993; 23: 3286-91.

316. Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell 1992; 69: 1227-36.

317. Kahan BD, Gibbons S, Tejpal N, Stepkowski SM, Chou T-C. Synergistic interactions of Cyclosporine and Rapamycin to inhibit immune performances of normal human peripheral blood lymphocytes in vitro. Transplantation 1991; 51: 232-8.

318. Calne RY, Lim S, Samaan A, St. J. Collier D, Pollard SG, White DJG, Thiru S. Rapamycin for immunosuppression in organ allografting. Lancet 1989; 2: 227.

319. Stepkowski SM, Chen H, Daloze P, Kahan BD. Prolongation by Rapamycin of heart, kidney, pancreas, and small bowel allograft survival in rats. Transplant Proc 1991; 23(1): 507-8.

320. Stepkowski SM, Chen H, Daloze P, Kahan BD. Rapamycin, a potent immunosuppressive drug for vascularized heart, kidney, and small bowel transplantation in the rat. Transplantation 1991; 51(1): 22-26.

321. Stepkowski SM, Chen HF, Wang ME, Daloze P, Kahan BD. Inhibition of host-versus-graft and graft-versus-host responses after small bowel transplantation in rats by Rapamycin. Transplantation 1992; 53(2): 258-64.

322. Fabian MA, Denning SM, Bollinger RR. Rapamycin suppression of host-versus-graft and graft-versus-host disease in MHC-mismatched rats. Transplant Proc 1992; 24(3): 1174.

323. Chen H, Wu J, Xu D, Aboujaoude M, Stepkowski S, Kahan B, Daloze P. The effect of rapamycin on orthotopic small bowel transplantation in the rat. Transplant Proc 1992; 24(3): 1157-8

324. Morris RE, Meiser BM, Wu J, Shorthouse R, Wang J. Use of Rapamycin for the suppression of alloimmune reactions in vivo: Schedule dependence, tolerance induction, synergy with cyclosporine and FK506, and drrect on host-versus-graft and graft-versus-host reactions. Transplant Proc 1991; 23(1): 521-4.

325. Almond PS, Moss A, Nakhleh R, Melin M, Chen S, Salazar A, et al. Rapamycin: Immunosuppression, hyporesponsiveness, and side effects in a porcine renal allograft model. Transplantation 1993; 56(2): 275-81.

326. Yatscoff RW, LeGatt DF, Kneteman NM. Therapeutic monitoring of rapamycin: A new immunosuppressive drug. Ther Drug Monit 1993; 15(6): 478-82.

327. Ochiai T, Gunji Y, Nagata M, Komori A, Asano T, Isono K. Effects of Rapamycin in experimental organ allografting. Transplantation 1993; 56(1): 15-19.

328. St. J. Collier D, Calne RY, Pollard SG, Friend PJ, Thiru S. Rapamycin in experimental renal allografts in primates. Transplant Proc 1991; 23(4): 2246-7.

329. DiJoseph J, Mihatsch M, Sehgal S. Influence of rat strain on rapamycin's kidney effects. Transplant Proc 1993; 25(1): 6714-5.

330. Whiting PH, Woo J, Adam BJ, Hasan NU, Davidson RJL, Thomson AW. Toxicity of Rapamycin - A comparative and combination study with Cyclosporin at immunotherapeutic dosage in the rat. Transplantation 1991; 52(2): 203-8.

331. Makowka L, Svanas G, Esquivel C, *et al.* The effect of Cyclosporine on hepatic regeneration. Surg Forum 1986; 37: 352.

332. Francavilla A, Starzl TE, Scotti C, Carrieri G, Azzarone A, Zeng QH, *et al.* Inhibition of liver, likney, and intestine regeneration by Rapamycin. Transplantation 1992; 53(2): 296-8.

333. Takeuchi T, Iinuma H, Kunimoto S, Masuda T, Ishizuka M, Takiuchi M. A new antitunor antibiotic, spergualin: Isolation and antitumor activity. J Antibiot 1981; 34: 1619-21.

334. Umezawa H, Ishizuka M, Takeuchi T, Abe F, Nemoto K, Shibuya K, Nakamura T. Suppression of tissue graft rejection by spergualin. J Antibiot 1985; 38: 283-4.

335. Suzuki S, Kanashiro M, Watanabe H, Sakakiraba I, Amemiya H. Therapeutic effects of 15-Deoxyspergualin on acute graft rejection detected by ^{31}P nuclear magnetic resonance spectrography, and its effect on rat heart transplantation. Transplantation 1988; 46(5): 669-72.

336. Suzuki S, Kanashiro M, Watanabe H, Sakakiraba I, Amemiya H. Effect of 15-Deoxyspergualin on rejection detected by ^{31}P nuclear magnetic resonance spectroscopy and in vivo mechanism of action in rat heart transplantation. Transplant Proc 1989; 21(1): 1094-5.

337. Jiang H, Takahara S, Takano Y, Machida M, Iwasaki A, Kokado Y, *et al.* In vitro immunosuppressive effect of Deoxymethylspergualin. Transplant Proc 1990; 22(4): 1633-7.

338. Nishimura K, Tokunaga T. Effects of 15-Deoxyspergualin on the induction of cytotoxic T lymphocytes and bone marrow suppression. Transplant Proc 1989; 21(1): 1104-7.

339. Kerr PG, Atkins RC. The effects of deoxyspergualin on lymphocytes and monocytes in vivo and in vitro. Transplantation 1989; 48: 1048-52.

340. Gannedahl G, Karlsson-Parra A, Totterman TH, Tufveson G. 15-Deoxyspergualin inhibits antibody production in mouse to rat heart transplantation. Transplant Proc 1993; 25(1): 778-80.

341. Nadler SG, Tepper MA, Schacter B, Mazzucco CE. Interaction of the immunosuppressant deoxyspergualin with a member of the Hsp70 family of heat shock proteins. Science 1992; 258: 484-6.

342. Suzuki CK, Bonifacino JS, Lin AY, Davis MM, Klausner RD. Regulating the retention of T-cell receptor α chain variants within the endoplasmic reticulum: Ca^{2+}-dependent association with BiP. J Cell Biol 1991; 114: 189-205.

343. VanBuskirk AM, DeNagel DC, Guagliardi LE, Brodsky FM, Peirce SK. Cellular and subcellular distribution of PBP72/74, a peptide-binding protein that plays a role in antigen processing. J Immunol 1991; 146: 500-6.

344. Nemoto K, Mayashi M, Abe F, Nakamura T, Ishizuka T, Umezawa H. Immunosuppressive activities of 15-deoxyspergualin in animals. J Antibiot 1987; 40: 561.

345. Yuh DD, Morris RE. 15-Deoxyspergualin is a more potent and effective immunosuppressant than Cyclosporine but does not effectively suppress lymphoproliferation in vivo. Transplant Proc 1991; 23(1) 535-9.

346. Schubert G, Stoffregen C, Looke G, Timmermann W, Schung T, Thiede A. Synergistic effect of 15-deoxyspergualin and cyclosporin A in pancreatic transplantation. Transplant Proc 1989; 21(1): 1096

347. Kamata K, Okubo M, Masaki Y, Inoue K, Umetani N, Sato N, Uchida H. Effect of 15-deoxyspergualin on the survival of thyroid allografts in mice. Transplant Proc 1989; 21(1): 1099-103

348. Von Specht B-U, Dibelius A, Konigsberger H, Lodde G, Roth H, Permanetter W. Prolongation of islet and pancreas graft survival in spontaneous diabetic BB/W rats by perioperative cyclosporin or 15-deoxyspergualin therapy. Transplant Proc 1989; 21(1): 965-7.

349. Koyama I, Kadokura M, Hoshino T, Omoto R. Effective use of 15-deoxyspergualin in kidney transplantation. Transplant Proc 1989; 21(1): 1088-9.

350. Niiya S, Suzuki S, Hayashi R, Watanabe H, Itoh J, Amemiya H. 15-deoxyspergualin: a powerful rescue drug for rejection in canine kidney grafting. Transplant Proc 1989; 21(1): 1076.

351. Morisaki F, Sakagami K, Orita K. Effect of 15-deoxyspergualin on class II antigen expression in hepatic sinusoidal lining cells and on infiltration of monomuclear cells in swine liver transplantation Transplant Proc 1989; 21(1): 1077-80.

352. Gannedahl G, Wanders A, Carlsson M, Tufveson G. 15-Deoxyspergualin effects in rat heart allograft transplantation--Relation to dose, timing, and cyclosporine. Transplantation 1993; 55(2): 455-6.

353. Gannedahl G, Wanders A, Carlsson M, Tufveson G. 15-Deoxyspergualin potentiates the immunosuppressive effect of Cyclosporine A in a rat heart allograft model. Transplant Proc 1993; 25(1) 768-9.

354. Suzuki S, Nishimori H, Kido K, Arita S, Tashiro H, Ohdan H, Quinones K, Amemiya H. Continuous administration of Methyldeoxyspergualin prolongs xenograft survival in hamster-to-rat cardiac transplantation. Transplant Proc 1993; 25(1): 430-1.

355. Tanaka S, Okada H, Uda K, Fujioka M, Makabe M, Orita T, Orita K. Mechanism of graft-versus-host disease and inhibition with deoxyspergualin on small bowel transplantation in rats. Transplant Proc 1992; 24(3): 1161-3.

356. Andreoni B, Biffi R, Derai P, Bardella MT, Velio P, Pozzi S, *et al*. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: P90.

357. [illegible]uki S, Hayashi R, Kenmachi T, Shimatani K, Fukuoka T, Ameniya H. Clinical application of 15-Deoxyspergualin for treatment of acute graft rejection following renal transplantation. Transplant Proc 1990; 22(4): 1615-17.

358. Okubo M, Tamura K, Kamata K, Tsukamoto Y, Nakayama T, Osakabe T, *et al.* 15-Deoxyspergualin "rescue therapy" for methylprednisolone-resistant rejection of renal transplants as compared with anti-T cell monoclonal antibody (OKT3). Transplantation 1993; 55(3): 505-8.

359. Amemiya H, Taguchi Y, Fukao K, Isono K, Omoto R, Ota K, *et al.* Establishment of rejection therapy with Deoxyspergualin by multicentral controlled clinical studies in renal recipients. Transplant Proc 1993; 25(1): 730-3.

360. Takahara S, Moutabarrik A, Kokado Y, Kameoka H, Takano Y, Zaid D, et al. Use of 15-Deoxyspergualin in treatment of acute rejection in kidney transplant patients. Transplant Proc 1993; 25(3): 2254-8.

361. Xenos E, Casanova D, Sutherland D, Farney A, Lloveras J, Gores P. The in vivo and in vitro effect of 15-Deoxyspergualin on pancreatic islet function. Transplantation 1993; 56(1): 144-7.

362. Jindal RM, Soltys K, Yost F, Beer E, Tepper MA, Cho SI. Effect of Deoxyspergualin on the endocrine fuention of the rat pancreas. Transplantation 1993; 56(5): 1275-7.

363. Gores PF, Najarian JS, Stephanian E, Lloveras J, Kelley S, Sutherland DER. Insulin independence in type I diabetes after transplantation of unpurified islets from single donor with 15-Deoxyspergualin. Lancet 1993; 341: 19-21.

364. Yuh DD, Morris RE. The immunopharmacology of immunosuppression by 15-Deoxyspergualin. Transplantation 1993; 55(3): 578-91.

365. Amemiya H, Suzuki S, Niiya S, Fukao K, Yamanaka N, Ito J. A new immunosuppressive agent, 15-deoxyspergualin in dog renal allograf.ing. Transplant Proc 1989; 21: 3468-70.

366. Fukao K, Iwasaki H, Yuzawa K, Otsuka M, Yu Y, Sharma N, *et al.* Immunosuppressive effect and toxicity of 15-Deoxyspergualin in cynanolgus monkey. Transplant Proc 1991; 23(1): 556-8.

367. Sweeney MJ, Hoffman DH, Esterman MA. Metabolism and biochemistry of mycophenolic acid. Cancer Res 1972; 32: 1803-9.

368. Platz KP, Sollinger HW, Hullett DA, Eckhoff DE, Eugui EM, Allison AC. RS-61443 -- a new, potent immunosuppressive agent. Transplantation 1991; 51(1): 27-31.

369. Morris RE, Hoyt EG, Murphy MP, Eugui EM, Allison AC. Mycophenolic acid morpholinoethylester (RS-61443) is a new imminosuppressant that prevents and halts heart allograft rejection by selective inhibition of T- and B-cell purine synthesis. Transplant Proc 1990; 22(4): 1659-62.

370. Allison AC, Eugui EM. Inhibitors of de novo purine and pyrimidine synthesis as immunosuppressive drugs. Transplant Proc 1993; 25(3)(supp 2): 8-18.

371. Franklin TJ, Cook JM. The inhibition of nucleic acid synthesis by mycophenolic acid. Biochem J 1969; 113: 515-24.

372. Burlingham WJ, Grailer AP, Hullett DA, Sollinger HW. Inhibition of both MLC and in vitro IgG memory response to tetanus toxoid by RS-61443. Transplantation 1991; 51(2): 545-7.

373. Allison AC, Kowalski W, Muller C, Waters R, Eugui EM. Mycophenolic Acid and Brequinar, inhibitors of purine and pyrimidine synthesis, block the glycosylation of adhesion molecules. Transplant Proc 1993; 25(3)(supp 2): 67-70.

374. Isobe M, Yagita H, Okumura K, Ihara A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. Science 1992; 255: 1125-7.

375. Bechstein WO, Schilling M, Steele D, Hullet D, Sollinger HW. RS-61443/Cyclosporine combination therapy prolongs canine liver allograft survival. Transplant Proc 1993; 25(1): 702-3.

376. Kawamura T, Hullett D, Suzuki U, Bechstein W, Allison A, Sollinger HW. Enhancement of allograft survival by combination RS-61443 and DUP-785 therapy. Transplantation 1993; 55(4): 691-5.

377. Shaffer D, Blakely ML, Gottschalk R, Monaco AP. Small bowel transplantation in rats using RS-61443: Effect on GVHD and rejection. Transplant Proc 1992; 24(3): 1159-60.

378. Sonnino RE. RS-61443 prevents graft-versus-host disease but not rejection in allogeneic rat small bowel transplants. Transplant Proc 1992; 24(3): 1190.

379. D'Alessandro AM, Rankin M, McVey J, Hafez GR, Sollinger HW, Kalayoglu M, Belzer FO. Prolongation of canine intestinal allograft survival with RS-61443, Cyclosporine, and Prednisone. Transplant Proc 1993; 25(1): 1207-9.

380. D'Alessandro AM, Rankin M, McVey J, Hafez GR, Sollinger HW, Kalayoglu M, Belzer FO. Prolongation of canine intestinal allograft survival with RS-61443, Cyclosporine, and Prednisone. Transplantation 1993; 55(4): 695-701.

381. Nakajima K, Ochiai T, Nagata M, Suzuki T, Gunji Y, Asano T, *et al.* Effects of triple therapy of Cyclosporine, FK506, and RS-61443 on allogeneic small bowel transplantation in dogs. Transplant Proc 1993; 25(1): 595-6.

382. Deierhoi MH, Sollinger H, Deithelm A, Belzer F, Kauffman RS. One-year follow-up results of a phase I trial of Mycophenolate Mofetil (RS61443) in cadaveric renal transplantation. Transplant Proc 1993; 25(1): 693-4.

383. Salaman JR, Griffin P, Johnson RWG, Kohlhaw K, Land W, Moore R, *et al.* Controlled trial of RS-61443 in renal transplant patients receiving Cyclosporine monotherapy. Transplant Proc 1993; 25(1): 695-6.

384. Sollinger H, Belzer FO, Deierhoe M, Diethelm A, Gonwa TA, Kauffman RS, *et al.* RS-61443: rescue therapy in refractory kidney transplant rejection. Transplant Proc 1993; 25(1): 698-9.

385. Klintmalm GB, Ascher NL, Busuttil RW, Deierhoi M, Gonwa TA, Kauffman R, *et al.* RS-61443 for treatment-resistant human liver rejection. Transplant Proc 1993; 25(1): 697.

386. Ensley RD, Bristow M, Olsen S, Taylor D, Hammond E, O'Connell J, *et al.* The use of Mycophenolate Mofetil (RS-61443) in human heart transplant recipients. Transplantation 1993; 56(1): 75-82.

387. Eugue EM, Almquist SJ, Muller CD, Allison AC. Lymphocyte-selective cytostatic and immunosuppressive effects of mycophenolic acid in vitro: role of deoxyguanosine nucleotide depletion. Scand J Immunol 1991; 33: 161-73.

388. Makowka L, Chapman F, Cramer D. Historical development of Brequinar Sodium as a new immunosuppressive drug for transplantation. Transplant Proc 1993; 25(3) (supp 2): 2-7.

389. Jaffee BD, Jones EA, Loveless SE,Chan SF. The unique immunosuppressive activity of Brequinar Sodium. Transplant Proc 1993: 25(3) (supp 2): 19-22.

390. Cramer DV, Chapman FA, Jaffee BD, Jones EA, Knoop M, Hreha-Eiras G, Makowka L. The effect of a new immunosuppressive drug, Brequinar Sodium, on heart liver, and kidney allograft rejection in the rat. Transplantation 1992; 53: 303-8.

391. Knoop M, Cramer D, Chapman F, Makowa L. Brequinar Sodium suppresses liver allograft rejection and .nduces permanent tolerance in the rat. Transplant Proc 1993; 25(1): 706-7.

392. Yasunaga C, Cramer D, Chapman F, Wang HK, Barnett M, Wu GD, Makowka L. Cardiac graft rejection in hypersensitized recipients: Prevention of antibody response and graft rejection using Brequinar Sodium. Transplant Proc 1993; 25(3) (supp 2): 65-6.

393. Cosenza CA, Cramer D, Eiras-Hreha G, Cajulis E, Wang H, Makowka L. Evaluation of the use of Brequinar Sodium and Cyclosporine combination therapy for preventing rat cardiac allograft rejection. Transplant Proc 1993; 25(3) (supp 2): 57-8.

394. Cosenza CA, Cramer D, Eiras-Hreha G, Cajulis E, Wang H, Makowka L. The synergism of Brequinar Sodium and Cyclosporine used incombination to prevent cardiac allograft rejection in the rat. Transplantation 1993; 56(3): 667-72.

395. Collins BH, Areford M, Fabian M, Jaffe B, Bollinger R. Brequinar Sodium potentiates the effects of Cyclosporine in experimental small bowel transplantation. Transplant Proc 1993; 25(3): 37-9.

396. Kahan BD, Tejpal N, Gibbons-Stubbers S, Tu Y, Wang M, Stepkowski S, Chou T-C. The synergistic interactions in vitro and in vivo of Brequinar Sodium with Cyclosporine or rapamycin alone and in triple combination. Transplantation 1993; 55(4): 894-900.

397. Stepkowski SM, Kahan BD. Synergistic activity of the triple combination: Cyclosporine, Rapamycin, and Brequinar. Transplant Proc 1993; 25(3) (supp 2): 29-31.

398. Barnes TB, Campbell P, Zajac I, Gayda D. Toxicological and pharmacokinetic effects following coadministration of Brequinar Sodium and Cyclosporine to Sprague-Dawley rats. Transplant Proc 1993; 25(3) (supp 2): 71-4.

399. Chong AS-F, Gebel H, Finnegan A, Petraitis EE, Jiang XL, Sankary HN, *et al.* Leflunomide, a novel immunomodulatory agent: In vitro analyses of the mechanism of immunosuppression. Transplant Proc 1993; 25(1): 747-9.

400. Williams AJW, Xiao F, Foster PF, Chong A, Sharma S, Bartlett R, Sankary H. Immunosuppressive effects of Leflunomide in a cardiac allograft model. Transplant Proc 1993; 25(1): 745-6.

401.. Schorlemmer HU, Seiler FR, Bartlett RR. Prolongatio of allogeneic transplanted skin grafts and induction of tolerance by leflunomide, a new immunosuppressive isoxazole derivative. Transplant Proc 1993; 25(1): 763-7.

402. Barlett RR, Dimitrijevic M, Mattar T, Zielinski T, Germann T, Rude E, *et al.* Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection. Agents Actions 1991; 32: 10-21.

403. He G, McAllister V, Lee T, Bitter-Suermann H, Theal M, Wright J, MacDonald A. Oral Leflunomide prevents rejection following rat small bowel transplantation. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O97.

404. Foster PF, Xiao F, Kociss K, Sankary HN,McChesney L, Williams JW. Leflunomide immunosuppression in rat small intestinal transplantation. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: P95.

405. Bowles MJ, Webster GA, Wood RFM, Pockley AG. Effect of anti-CD4 monoclonal antibody therapy on rat small bowel allograft survival. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: P98.

406. Stangl MJ, Lee KKW, Lee TK, Moynihan HL, Schraut WH. Graft pretreatment with monoclonal antibodies prior to small-bowel transplantation. Transplant Proc 1990; 22(6): 2483-4.

407. Davies CB, Madden RL, Alexander JW, Cofer BR, Fisher RA, Anderson P. Effect of a short course of Rapamycin, Cyclosporin A, and donor-specific transfusion on rat cardiac allograft survival. Transplantation 1993; 55(5): 1107-12.

408. Cofer BR, Tchervenkov JI, Alexander JW, Barnwell SC. Donor specific transfusion and cyclosporine prolong allograft survival when given in the preoperative period. Transplant Proc 1991; 23(1): 157-8.

409. Martinelli GP, Chung-Loy R, Sher L, Racels D, Miller CM, Schanzer H. Long-term prolongation of cardiac allografts by subtherapeutic levels of cyclosporine in rats conditioned with pretranplant blood transfusions and cyclosporine. Transplantation 1985; 39(1): 1-5.

410. Fecteau A, Tchervenkov J, Guttman FM, Rosenmann E. Small bowel transplantatio in the rat: The effect of donor-specific transfusion 24 hours pretransplant and Cyclosporine. Transplant Proc 1992; 24(3): 1166.

411. DeBruin RWF, Heineman E, Jeekel J, Marquet RL. The effect of donor-specific blood transfusion on orthotopic small-bowel transplantation in rats. Transplant Proc 1992; 22 (6): 2491-2.

412. Martinelli GP, Knight RK, Kaplan S, Racelis D, Dirkman SH. Schanzer H. Small bowel transplantation in the rat: Effect of pre-transplant blood transfusions and cyclosporine on host survival. Transplantation 1988; 45: 1021-6.

413. Takara T, Tchervehkov JI, Guttman FM, Fecteau A, Rosenmann E. Intraportal donor specific transfusions (DST) given 24hrs before small bowel transplantation with CsA and FK506. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: P79.

414. Astarcioglu I, Gugenheim J, Gigou M, Amorosa L, Fabiani B, Reynes M, Bismuth H. Immunosuppressive properties of auxiliary liver allografts into sensitized rats. Transplantation 1990; 49(6): 1186-8.

415. Greenstein SM, Sun SC, Senitzer D, Schechner RS, Tellis VA. Pretreatment with irradiation and donor-specific transfusion prolongs survival in small intestinal transplantation. Transplant Proc 1993; 25(1): 490-1.

416. Calne RY, Sells R, Pena JR, Davis DR, Millard PR, Herbertson BM, *et al.* Induction of immunological tolerance by porcine liver allografts. Nature 1969; 223: 472-6.

417. Kamada N, Davies HS, Roser B. Reversal of transplantation immunity by liver grafting. Nature 1981; 292: 840-2.

418. Kamada K. The immunology of experimental liver transplantation in the rat. Immunology 1985; 55: 369-89.

419. Gonwa TA, Nery JR, Husberg BS, Klintmalm GB. Simultaneous liver and renal transplantation in man. Transplantation 1988; 46(5): 690-3.

CHAPTER III

CYCLOSPORIN

INTRODUCTION

Ever since Lillehei demonstrated the technical feasibility of small bowel transplantation (SBT) (1), there has been an increasing interest in this procedure as a cure for short bowel syndrome. However, as discussed in the introduction, most attempts at SBT have had a poor outcome due to lack of adequate immunosuppression protocols. Graft rejection or complications of immunosuppression such as drug toxicity, overwhelming sepsis and the development of post-transplantation lymphoproliferative disorders continue to cause significant morbidity and mortality (2-4). The early era of experimental and clinical SBT utilized conventional immunosuppressive regimens involving 6-mercaptupurine azathioprine, corticosteroids and antilymphocyte sera (ALS) (5-8). With the discovery of cyclosporin A (CsA) and its successes in other organ transplantation, however, it has now become a key agent for both experimental and clinical SBT.

In a 1981 landmark study by Reznik *et al*, it was demonstrated that CsA could prolong the survival of small bowel allografts in dogs with chronic doses of 25 mg/kg/d, intramuscular (i.m.) and *per os* (p.o.) (9). Since then, the rat model has become the prototypical animal model for SBT because of the availability of inbred strains with which to study rejection responses. In rats, CsA can prolong survival of completely allogeneic small bowel transplants with a wide array of doses ranging from 20 mg/kg/d indefinitely, to 15 mg/kg/d for just 2 weeks, to 15 mg/kg/d for 1 week followed by every alternate day for 4 to 5 weeks, (10-13) to 5 mg/kg/d for only 2 weeks (14, 15).

Allograft acceptance, however, is not the only requirement for the success of SBT. The absorptive function of the transplanted intestine must also be adequate. Studies in the rat model suggest that the

actual process of transplantation may adversely affect the uptake of electrolytes, glucose, glycine and other nutrients, especially fat, by the disruption of lymphatics, denervation and possibly changes in villus surface area (12, 16-25). Adverse effects presumed secondary to immunosuppression have also been demonstrated (17, 26, 27). However, these studies have been performed on transplanted bowel, either isografts or allografts, such that any immunological or mechanical consequences of transplantation may confound the results, making the interpretation of effects directly attributable to immunosuppression difficult to assess. Our group has previously shown that CsA negatively affects nutrient absorption and increases intestinal permeability in normal (non-transplanted) bowel (28-30). This has prompted the present study, where we wished to further examine these effects of CsA on small bowel function, in the context of growth and nutrient absorption.

MATERIALS AND METHODS

ANIMALS

Juvenile male Lewis rats (275-300 g) were obtained from Charles River Canada, St. Constant, P.Q., and housed in individual plexiglass cages with free access to water and standard lab rat chow (Lab Diet, PMI Feeds, St. Louis, MO.) with an approximate composition of 23.0% protein, 4.5% fat, 6.0% fiber, 8.0% ash and 2.5% minerals. Day/night cycles were 12 hours and the temperature was maintained at 20 ± 2°C. Feed intake was monitored weekly, and body weight changes biweekly. Animal care was in accordance with the guidelines of the Canadian Council of Animal Welfare (31). The experimental protocol was approved by the Animal Welfare Committee of the University of Alberta.

EXPERIMENTAL GROUPS

The CsA-treated animals (n=10) received a typical immunosuppression protocol of CsA, 15 mg/kg/d, dissolved in medium chain triglyceride oil (MCT), injected subcutaneously (s.c.) into the nape of the neck for 6 days, followed by the same dose on alternate days for 5 more weeks. Previous studies in our laboratory have shown this dose and schedule to give therapeutic drug levels (9,12). Control animals

received a corresponding volume/kg of the drug vehicle., s.c., on the same schedule. As discussed in Chapter I of this thesis, the complete study undertaken was performed in two sets of experiments. This subsequently provided two control groups against which the test group could be compared: "control 1" (n=10) which was studied alongside CsA in the first set of experiments, and an overall control group of n=20, consisting of "control 1" and "control 2". The latter (n=10) was utilized during the second set of experiments. Each control group was divided in half, with 5 animals receiving MCT oil, and 5 receiving carboxymethylcellulose (CMC), the vehicle used with some of the other drugs studied.

DRUGS

CsA (Sandimmune®; a generous gift of Sandoz Pharmaceutical Corp., Montreal) was dissolved in MCT oil (Mead-Johnson, Ottawa, Ontario) to a concentration of 18 mg/ml, and sterilized by microfiltration. It was prepared every four weeks and stored in the dark at 4°C. Drug concentrations were prepared so that equivalent volumes (0.83 ml/kg) were injected throughout the study. Control animals received equivalent volumes per kg of the liquid vehicles, also sterilized by microfiltration.

***IN VIVO* NUTRIENT BALANCE STUDIES**

After 33 days of treatment, the animals were placed in individual metabolic cages, preconditioned for 3 days, and then underwent a 3 day balance study with daily measurement of food-intake and fecal collection. Standard lab rat chow was given *ad lib*, with a composition as described above. Total carbohydrate and fat content of feed and feces were determined using standard methods (32, 33). Briefly, feed and collected feces were completely desiccated in a freeze-dryer, and the percentage of water determined directly by the change in total weight. Dried feces were then ground into a powder, and 0.2 g of sample used to determine the total energy content using a Gallenkamp Ballistic Bomb Calorimeter (London, England). 0.25 g of sample was then rehydrogenated and the fat extracted into the solvent layer of a 0.9% sodium-chloride-chloroform:methanol (2:1) extraction mixture. After a second extraction of the aqueous layer, to ensure completeness, the solvent was evaporated off and the final contents weighed to

determine the amount of total fat extracted. From these results, the percentages of energy and fat absorption from feed intake were calculated directly.

HEMATOLOGICAL PARAMETERS

Serum electrolytes and creatinine were determined at sacrifice using a Beckman Astra-Eight analyzer.

DRUG LEVELS

CsA whole blood levels were drawn after two weeks of drug therapy and at sacrifice. Peak levels (drawn two hours post-injection) and trough levels (drawn 24 hours post-injection) were determined by HPLC, which specifically measures only the parent drug (34).

***IN VIVO* INTESTINAL PERMEABILITY**

In the fifth week of the study, animals were fasted overnight and then gavaged with 100 mg of mannitol and 100 mg of lactulose in 2 ml of water during which time the animals were allowed free access to water but not food. Urine was quantitatively collected and urinary recovery of each compound was measured using high performance liquid chromatography and reported as both the percentage recovery of the total oral dose of each carbohydrate probe, and the ratio of lactulose to mannitol recovered. This latter measurement is felt to best reflect the permeability of the bowel per unit surface area (35, 36).

VILLUS MORPHOMETRY AND DENSITY

At sacrifice, sections of jejunum and ileum from control and test animals were fixed in formalin and mounted in paraffin blocks. They were cut so that both longitudinal and cross-sections of bowel were obtained which were then stained with haematoxylin and eosin. As an indirect measurement of mucosal surface characteristics, sections of ileum and jejunum, for each group, were photomicrographed at a magnification of 100X. Morphometric analyses were performed using an Image analysis system (Joyce Loebl, Magiscan) (37). Image analysis systems have been used to efficiently determine morphometric parameters of large numbers of intestinal villi in several recent studies analyzing intestinal mucosal architecture (38, 39). Parameters studied included villus length, as measured from the lamina propria,

width and overall sagittal section area for 25 villi per histological section. Three sections from both the jejunum and ileum for each test group and four sections from both the jejunum and ileum of the controls were analyzed, resulting in 75 to 100 villi being measured per group for each region of small bowel. The number of villi per microscopic field (at 20X magnification) were determined as a measure of villus density.

IN VITRO GLUCOSE FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

After 6 weeks of treatment, animals were sacrificed with an intraperitoneal pentobarbital overdose and *in vitro* small bowel function studies were performed. Segments of distal ileum and proximal jejunum, taken approximately 2 to 3 cm from the ileocecal valve and ligament of Trietz, respectively, were quickly excised, rinsed with ice-cold normal Ringers solution, and stored in ice-cold Ringers gassed with carbogen (5% CO_2 in O_2) during the preparation for mounting onto Ussing chambers as previously described (40, 41). Briefly, the intestine was split along the mesenteric border and segments of approximately 2 cm were then quickly stripped of the serosa and underlying muscle layers and then clamped into Ussing chambers . This subsequently divides the chamber into mucosal and serosal compartments. Tissues were bathed on both sides by normal Ringers (Na^+, 143 mM; K^+, 5 mM; Ca^{2+}, 1.25 mM; Mg^{2+} 1.1 mM; Cl^-, 123.7 mM; HCO_3, 25 mM; $HPO_4 + H_2PO_4$, 1.95 mM) with 20 mM fructose, and gassed with carbogen to pH of 7.4. A circulating water bath maintained the chamber temperature at 37°C. For measurement of unidirectional fluxes, 3-O-methyl-D-glucose (3OMeG) was present at a concentration of 20 mM on both mucosal and serosal sides and 5 μCi of [^{3}H]-3OMeG was added to either the mucosal or serosal side of the tissue. To ensure steady-state conditions, tissues were maintained under these conditions for 20 minutes. Previous studies have shown that glucose flux is constant over this time period (41). Unidirectional mucosal-to-serosal (J_{ms}) and serosal-to-mucosal (J_{sm}) fluxes were measured in paired tissues by collection of aliquots of incubation solution from each side of the chambers and determining the radioactivity in a Beckman LS5801 β-Scintillation Counter (California). Tissue pairs were discarded if electrical conductance (see below) varied by >15%. Four

consecutive 5 minute fluxes were determined. Tissue response to 5 mM theophylline was used to confirm viability at the completion of the experiment.

Electrophysiological parameters were monitored throughout. The spontaneous transepithelial electrical potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (I_{sc}) with an automatic voltage clamp (DCV 1000 World Precision Instruments, New Haven, CT), except for 3-5 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue conductance (G) was calculated from potential difference and I_{sc} according to Ohm's law, where voltage (V) = current (I_{sc}) x resistance (R) and conductance (G) is the reciprocal of R (40).

STATISTICAL ANALYSES

Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using a computerized statistical package (SigmaStat Statistical Analysis System, Jandel Corporation). Means between the two groups (controls vs treatment) were compared using the Student's t-test unless the distribution was nonparametric (as determined directly by the program, using the Kolmogorov-Smirnov test of normality, with a p-value of >0.05) in which case a distribution-free test comparing the medians is more appropriate, and was performed with a Mann-Whitney rank sum test. A p-value of <0.05 was considered statistically significant. No statistically significant differences were determined between the MCT and CMC treatments, and in all but three sets of results (stool water content, lactulose/mannitol permeability and villus morphometry), no differences were found between the two control groups ("control 1" and "control 2"). Subsequently, all control animals were combined into one control group with n=20. In studies where the two significantly differed, the differences were assumed to be secondary to altered laboratory processing of the samples, and the CsA group was compared to its respective "control 1" group, only.

RESULTS

WEIGHT GAIN, FEED INTAKE & *IN VIVO* NUTRITIONAL BALANCE STUDIES

Body weight gain over the six week period was not significantly affected in the CsA-treated animals despite a significant 7% increase in feed intake compared to controls. Energy (kcal) and fat absorption were significantly reduced by 4% and 10%, respectively. These effects are tabulated in Table III-1.

OTHER EFFECTS

Animals appeared well and active throughout the study. Neither significant diarrhea nor constipation developed in these animals. Stool water content (34.7 ± 1.3%) was increased by 13% relative to controls (water content of 30.6 ± 2.4%), but this was not statistically significant (p=0.168).

SERUM & HEMATOLOGICAL PARAMETERS

Serum K^+ was significantly increased by almost two-fold in the CsA-treated animals while creatinine was unchanged (Table III-2). The samples were extremely hemolyzed at the time of analysis, however, and thus, this is unlikely a manifestation of drug-induced nephrotoxicity.

DRUG LEVELS

The blood samples obtained for drug levels were extremely clotted. Nonetheless, mean peak and trough levels obtained after two weeks of drug administration were 1804 ± 637 ng/ml and 1919 ± 758 ng/ml, respectively. At sacrifice, the peak and trough levels were 1907 ± 345 ng/ml and 2181 ng/ml, respectively. The differences between levels at the different time periods were not statistically significant (p=0.812 between peak levels and p=0.668 between trough levels).

IN VIVO BOWEL PERMEABILITY

CsA-treated animals demonstrated a 25% decrease in mannitol recovery relative to controls. However, because of a 15% decrease (not statistically significant) in lactulose recovery, the lactulose/mannitol ratio was comparable to the controls (Table III-3).

VILLUS MORPHOMETRY AND DENSITY

Significant increases in villus width, height, and sagittal section area were observed throughout the bowel in the CsA-treated animals, compared to controls. This was reflected more in the jejunum, with parameter increases of 18% to 46% ($p<0.001$ for all values); increases of 9% to 14% were recorded in the ileum ($p<0.03$ for all values). Villus density, however, was unchanged. These changes are outlined in Table III-4 and displayed in Plates 1 and 2 (Appendix I).

IN VITRO 3OMeG FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

The effects of CsA on *in vitro* 3OMeG transmural fluxes are shown in Figure 4 and summarized in Table III-5. No significant differences compared to controls were noted in the jejunum. In the ileum, however, a 52% increase in J_{sm} was accompanied by a 33% increase in J_{ms}, with no subsequent change in J_{net}. This increase in J_{sm} corresponded with a two-fold increase in conductance and an associated 28% decrease in I_{sc} recorded in this region of small bowel.

DISCUSSION

As shown previously (28-30), CsA alters various parameters of nutrition and small bowel function when administered over a chronic dosing schedule, in normal (non-transplanted) rats. Significant reductions in *in vivo* fat and energy absorption were observed. These changes were not due to a reduction in mucosal surface area available for absorption. Rather, CsA had a hypertrophic effect on villus morphometry in both the jejunum and ileum, with no change in villus density. This has been reported previously (29). Interestingly, Heeckt *et al* have recently reported that CsA induces a cellular hyperplasia of enteric smooth muscle with no alterations of contractile function after 90 days of treatment (42). The mechanisms of this may be related to the gingival hyperplasia known to be caused by CsA, which occurs secondary to an increase in the numbers of mucosal collagen fibroblasts with increased production of collagen (43,44).

The reduction in fat absorption may be secondary to CsA's known cholestatic effects in rats which could affect the emulsification and subsequent absorption of dietary fats (45-47). This adverse effect on bile secretion does not appear to be due to direct effects on liver function enzymes, nor light or electron microscopic hepatic architecture (45,46,48). Rather, it appears to be secondary to CsA's inhibitory effect on the ATP-dependent bile salt transporter in the liver canalicular membrane (49-51).

The decrease in total energy absorption we observed was small, and may be the result of a combination of effects. It may be related in part to reduced active uptake of sugars by the small intestine We have previously shown that chronic administration of CsA in rats can alter the apparent kinetics of glucose uptake, resulting n a decrease of the V_{max} and increase of the K_m (29). Inhibition by CsA on the Na^+, K^+-ATPase pump of the enterocyte basolateral membrane, similar to its effects on the biliary canalicular membrane (49) and renal tubular cells (52), may reduce active glucose uptake However, as will be discussed below, active glucose uptake was not inhibited during *in vitro* 3OMeG flux studies. Alternatively, it is possible that some degree of protein malabsorption may account for some of the caloric loss in the stools. Although we did not look directly at protein absorption, and indeed, have found previously that CsA has little effect on protein absorption (28), others have found that CsA can produce a reversible reduction in neutral amino acid absorption during perfusion experiments in intestinal autografts (26). Uptake of neutral amino acids by the enterocyte occurs in a similar manner as glucose, also driven by the electrochemical Na^+ gradient maintained by the Na^+, K^+-ATPase at the basolateral membrane (53) It is possible, therefore, that CsA may inhibit this process of amino acid uptake and protein absorption

Looking directly at the *in vitro* glucose fluxes (Table III-3), the control animals demonstrated unidirectional and net glucose fluxes which were similar to those previously described in normal Lewis rats (41), confirming that chronic injection of the vehicles used did not have any significant effects on the flux or electrophysiological parameters studied. Treatment with CsA, however, induced increases in unidirectional flux in both the mucosal-to-serosal ($J_{m,s}$) and serosal-to-mucosal ($J_{s,m}$) directions throughout the bowel, but statistically significant in the ileum, only. These corroborate previous reports by our group

(17). The reasons for these changes are still not understood, but may be secondary to alterations in intestinal permeability (17,30). The increased conductance observed in the present study would support this hypothesis, whereby an increase in back diffusion of glucose from the serosal to mucosal sides of the bowel may result in a relative increase of glucose present at the brush border membrane. This, in turn, may stimulate a second messenger system to induce up-regulation of active glucose transport and/or increase paracellular movement of glucose to the serosal side, resulting in enhanced glucose absorption (54).

The effects of CsA on intestinal permeability in these and other studies are mixed. The *in vitro* increases in electrical conductance indicate that permeability may be increased, however, the *in vivo* results are more difficult to interpret. Intestinal permeability is normally the result of two types of pathways or physiologic breaks in the intestinal epithelium: the transcellular pathway or trans-membrane pores, and the paracellular pathway, regulated by the tight junctions. The latter is felt to be the major site for passive transepithelial permeation and implicated in changes in intestinal permeability (55). Larger probes such as Lactulose (molecular weight 342), ^{51}Cr-EDTA (mol wt., 292) and ^{99}Tc-DTPA (mol wt. 393) (56) permeate via gaps in the tight junctions. Smaller molecules such as mannitol (mol wt 182) can traverse both the transmembrane pores and tight junctions (57), and when administered simultaneously with a larger probe such as lactulose, can act as a marker for the rate of probe movement through the gastrointestinal tract, such that the ratio of recovery of lactulose to mannitol would be a more accurate indication of *in vivo* permeability (35,36).

CsA treated animals demonstrated a decrease in recovery of both lactulose and mannitol probes relative to controls. However, this may reflect an increase in gut motility, as suggested by the decrease in mannitol absorption. The lactulose/mannitol ratio in the test animals was comparable to controls, indicating no change in permeability and contrasting the *in vitro* increase in conductance. It also contrasts with previous studies showing an increase in *in vivo* ^{51}Cr-EDTA recovery in normal rats treated with CsA (30).

In order to resolve these inconsistencies, further *in vitro* studies need to be done, looking at *in vitro* permeability of these individual probes in Ussing chambers. Such studies would have the advantage of avoiding apparent variations in permeability due to *in vivo* variations in intestinal transit, mucosal water flux, blood and lymphatic flow, renal function and excretion and tissue or luminal metabolism of the probes (57).

Finally, the CsA whole blood levels must be commented on. The values obtained were certainly high, being approximately four times greater than the recommended trough values for humans (58,59). This was somewhat surprising, though, as the same mode of drug preparation and administration, with identical dosage and schedule, was utilized as had previously been reported by our laboratory, where CsA levels were within a more appropriate range of 311 μg/ml (trough) to 368 μg/ml (peak) (28). Possible explanations may involve processing of the blood samples, as they were extremely clotted at the time of procurement. Alternatively, the stock of CsA used was different than used previously by our laboratory as was the method of drug analysis, which was previously by radioimmunoassay (28). Most importantly, no significant toxicity's were seen as would be expected with a chronically toxic level, indicating that the true drug concentrations were most likely below the toxic level.

In summary, CsA, at the dose used, had little affect on growth and animal well-being in normal rats. Nutrient absorption was significantly compromised with respect to fat and energy, however these changes were small and not reflected by any changes in growth. They could not be accounted for by any reduction in mucosal surface area, which was, instead, increased to a small degree secondary to villus hypertrophy. At the enterocyte level, no changes in net 3OMeG uptake occurred despite CsA's proposed inhibitory effect on Na^+, K^+-ATPase function at the basolateral membrane. CsA may have increased intestinal permeability to a small degree, however, these changes were small, seen only *in vitro*, and did not result in any decrease in net 3OMeG mucosal-to-serosal flux. Barring the other adverse effects known to be caused by CsA, it could be considered as a potential agent to use in clinical small bowel transplantation, recognizing these limits on nutrient absorption and the potential implications when used in a patient who

may already be somewhat nutritionally compromised secondary to long standing short bowel syndrome and the process of intestinal transplantation itself.

Table III-1

Effects of Cyclosporin on Weight gain, Feed Intake and Energy and Fat Absorption

	Weight Gain (g/d)	Feed Intake (g/kg/d)	% Energy Absorption	% Fat Absorption
Control (n=20)	3.15 ± 0.07	69.42 ± 1.11	81.3 ± 0.6	76.1 ± 0.9
Cyclosporin (n=10)	2.95 ± 0.11 6%↓ p = 0.364	74.52 ± 0.84 ***7%↑** p < 0.001	78.3 ± 0.6 ***4%↓** p < 0.001	68.3 ± 0.9 ***10%↓** p < 0.001

Values are means ± SEM

***** significant difference compared to controls ($p<0.05$); compared with student's t-test.

% values indicated relative to controls (↑, increase; ↓, decrease).

Table III-2

Effects of Cyclosporin on Serum Electrolyte Values

	Na^+ (mmol/l)		K^+ (mmol/l)		Creatinine (mmol/l)	
Control 1 (n=10)	142.7 ± 0.9		9.3 ± 0.9		0.63 ± 0.02	
Cyclosporin (n=10)	136.3 ± 0.5	* **4%↓** p=0.001	17.6 ± 1.2	* **88%↑** p<0.001	0.43 ± 0.14	33%↓ p=0.11

Values are means ± SEM

* significant difference compared to controls ($p<0.05$); compared with student's t-test

% values indicated relative to controls (↑, increase; ↓, decrease).

Table III-3

Effects of Cyclosporin on *In Vivo* Intestinal Permeability

	Mannitol	Lactulose	Lactulose/ Mannitol
Control 1 (n=10)	3.77 ± 0.22	1.40 ± 0.11	0.41 ± 0.03
Cyclosporin (n=10)	2.84 ± 0.24 ***25%↓** p<0.013	1.19 ± 0.07 15%↓ p 0.11	0.43 ± 0.03 4%↑ p 0.668

Data is presented as percent recovery of orally administered marker in urine; mean ± SEM
* significant difference compared to controls ($p<0.05$); compared with student's t-test
% values indicated relative to controls (↑, increase; ↓, decrease).

Table III-4
Effects of Cyclosporin on Villus Morphometry and Density

	Width ($\mu m \times 10^2$)	Height ($\mu m \times 10^2$)	Area ($\mu m^2 \times 10^2$)	# Villi per 20X field
JEJUNUM				
Control 1 (n=10)	1.66 ± 0.05	4.74 ± 0.12	4.67 ± 0.19	6.58 ± 0.52
Cyclosporin (n=10)	1.96 ± 0.06 ***18%↑** p<0.001	5.74 ± 0.08 ***21%↑** p<0.001	6.81 ± 0.28 ***46%↑** p=0.601	5.61 ± 0.04 15%↓ p=0.139
ILEUM				
Control 1 (n=10)	1.70 ± 0.06	3.84 ± 0.14	4.13 ± 0.23	5.26 ± 0.41
Cyclosporin (n=10)	1.88 ± 0.06 ***11%↑** p=0.031	4.17 ± 0.06 ***9%↑** p=0.003	4.69 ± 0.18 ***14%↑** p=0.029	5.20 ± 0.23 1%↓ p=0.902

Data is presented as villus width, height from lamina propria and saggital section area; expressed as mean ± SEM
* significant difference compared to controls ($p<0.05$); compared with student's t-test
% values indicated relative to controls (↑, increase; ↓, decrease)

Table III-5
Effects of Cyclosporin on *In Vitro* 3OMeG Fluxes and Electrophysiological Parameters

	J_{ms}	J_{sm}	J_{net}	PD	I_{sc}	G
JEJUNUM						
Control (n=20)	1.24 ± 0.08	0.84 ± 0.06	0.41 ± 0.05	1.70 ± 0.12	39.29 ± 3.16	22.56 ± 1.54
Cyclosporin (n=10)	1.51 ± 0.16 22%↑ p=0.093	0.94 ± 0.07 12%↑ p=0.285	0.57 ± 0.12 39%↑ p=0.157	2.07 ± 0.11 21%↑ p=0.119	47.60 ± 3.69 21%↑ p=0.123	24.95 ± 2.11 11%↑ p=0.379
ILEUM						
Control (n=20)	1.19 ± 0.08	0.82 ± 0.08	0.36 ± 0.06	2.26 ± 0.15	40.86 ± 2.59	18.25 ± 1.50
Cyclosporin (n=10)	1.58 ± 0.11 ***33%↑** p=0.048	1.25 ± 0.11 ***52%↑** p=0.006	0.33 ± 0.05 8%↓ p=0.717	0.88 ± 0.16 ***61%↓** p<0.001	29.45 ± 4.33 ***28%↓** p=0.029	36.18 ± 3.31 ***98%↑** p<0.001

Values are means ± SEM. J_{ms}, flux from mucosa to serosa; J_{sm}, flux from serosa to mucosa; J_{net}, numerical difference between J_{ms} and J_{sm} in μ mol/cm^2/hr; PD, potential difference in mV; Isc, short-circuit current in μAmp/cm^2/hr; G, conductance in mS/cm^2.
*significant difference compared to controls ($p<0.05$); compared with student's t-test;% values indicated relative to controls (↑, increase; ↓, decrease).

REFERENCES

1. Lillehei RC, Idezuki Y, Feemster JA, Dietzman RH, Kelly WD, Merkel FK, *et al.* Transplantation of stomach, intestine and pancreas: Experimental and clinical observations. Surgery 1967; 62(4): 721-41.

2. Grant D, Wall W, Ghent C, Atkinson P,Williams S, Garcia B, *et al.* Small bowel transplantation at university Hospital, London, Ontario, Canada. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O3.

3. Todo S, Tzakis A, Reyes J, Abu-Elmagd K, Furukawa H,. Nour B, *et al.* Clinical intestinal transplantation: three-year experience. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O2.

4. Foster PF, Sankary HN, McChesney L, Xiao F, Kociss K, Koukoulis J, *et al.* Splanchnic transplantation. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O5.

5. Kahan BD. Transplantation timeline; Mankind's three millennia - one maverick's three decades in the struggle against biochemical individuality. Transplantation 1991; 51(1): 1-13.

6. Alican F, Hardy JD, Cayirli M, Varner JE, Moynihan PC, Turner MD, Anas P. Intestinal transplantation: Laboratory experience and report of a clinical case. Am J Surg 1971; 121: 150-9.

7. Ruiz JO, Lillehei RL. Intestinal transplantation. Am J Proctol 1972: 23; 379-93.

8. Fortner JG, Sichuk G, Litwin S, Beattie EJ, Jr. Immunological responses to an intestinal allograft with HL-A-identical donor-recipient. Transplantation 1972; 14(5): 531-5.

9. Reznick RK, Craddock GN, Langer B, Gilas T, Cullen JB. Structure and function of small bowel allografts in the dog: Immunosuppression with Cyclosperin A. Can J Surg 1982; 25(1): 51-5.

10. Harmel RP, Jr, Stanley M. Improved survival after allogeneic small intestinal transplantation in the rat using Cyclosporine immunosuppression. J Ped Surg 1986; 21(3): 214-7.

11. Lee KKW, SchrautWH. Structure and function of orthotopic small bowel allografts in rats treated with Cyclosporine. Am J Surg 1986; 151: 55-60.

12. Schraut WH, Lee KKW, Sitrin M. Recipient growth and nutritional status following transplantation of segmental small-bowel allografts. J Surg Res 1987; 43: 1-9.

13. Schraut WH, Lee KKW. Long-term surival of orthotopic small-bowel allografts using cyclosporin A. In: Deltz E, Thiede A, Hamelmann H, eds. Small-Bowel Transplantation. Berlin, Heidelberg: Springer, 1986: 156.

14. Shimazu R, Grogan JB, Raju S. Long-term survival of orthotopic bowel allografts in the rat treated with short-term low-dose Cyclosporine. Transplantation 1988; 46(5): 673-7.

15. Shimazu R, Grogan JB, Raju S. Successful orthotopic total bowel allotransplantation in the rat utilizing low-dose cyclosporine therapy. Transplant Proc 1989; 21(1): 2877-8.

16. Lee KK, Hurst RD, Schraut WH. Enteral Cyclosporin A absorption following orthotopic small-bowel transplantation. Gastroenterology 1987; 92: 1497.

17. Sigalet DL, Kneteman NM, Fedorak RN, Kizilisik T, Madsen KE, Thomson ABR. Small intestinal function following syngeneic transplantation in the rat. 1993; Submitted.

18. Ballinger WF, Christy, MG, Ashby, WB. Autotransplantation of the small intestine: The effects of denervation. Surgery 1962; 52: 151.

19. Schroeder P, Sandforth F, Deltz E. Glucose absorption after heterotopic small-bowel transplantation. In: Deltz E, editor. Small Bowel Transplantation. Experimental and Clinical Fundamentals. Berlin: Springer, 1986: 74-8.

20. Schroeder P, Deltz E, Seifert J, Sandforth F, Thiede A. Absorptive capacity of the transplanted small bowel. Gut 1987; 28 (suppl 1): 275-9.

21. Watson AJM, Lear PA, Montgomery A, Elliot E, Dacre J, Farthing MJG, Wood RFM. Water, electrolyte, glucose, and glycine absorption in rat small intestinal transplants. Gastroenterology 1988; 94: 863-9.

22. Raju S, Fujiwara H, Grogan JB, Achord JL. Long-term nutritional function of orthotopic small bowel autotransplants. J Surg Res 1989; 46: 142-6.

23. Sigalet DL, Kneteman NM, Fedorak RN, Kizilisik AT, Thomson ABR. Intestinal function following allogeneic small intestinal transplantation in the rat. Transplantation 1992; 53(2): 264-71.

24. Quigley EMM, Thompson JS, Rose SG. The long-term function of canine jejunoileal autotransplants: Insights into allograft physiology. Transplant Proc 1992; 24(3): 1105-6.

25. Ishii H, Hashimoto N, Kitada T, Kuroki T, Utsnomiya J. Malabsorption after small-bowel transplantation. Hepato-Gastroenterol 1993; 40: 282-4.

26. Collin J, Dennison AR, Watkins RM, Millard PR, Morris PJ. Segmental small intestinal allografts: II. Inadequate function with Cyclosporine immunosuppression: Evidence of a protein-losing enteropathy. Transplantation 1987; 44(4): 479-83.

27. Greenstein SM, Sun SC, Schechner RS, Senitzer D, Tellis VA. Prolonged use of cyclosporine (CsA) produces reversible hemodynamic and absorptive alterations in the transplanted rat small intestine. In: abstracts of the 27th annual meeting of The Association for Academic Surgery; 1993 Nov 10-13; Pennsylvania: abst #33.

28. Sigalet DL, Kneteman NM, Thomson ABR. The effects of Cyclosporine on normal bowel. Transplantation 1991; 51(6):1296-1302.

29. Sigalet DL Kneteman NM, Thomson ABR. Reduction of nutrient absorption in normal rats by Cyclosporine. Transplantation 1992; 53(5):1103-7.

30. Sigalet DL, Kneteman NM, Simpson I, Walker K, Thomson ABR. Intestinal permeability after small intestinal transplantation and Cyclosporine treatment. Transp Proc 1992; 24(3):1120-1.

31. Canadian Council on Animal Care. Guide to the care and use of Experimental Animals. The Council, 1980.

32. 15. Association of Official Analytical Chemist (1986): Methods of analysis (23 Ed.). AOAC, Washington, D.C.

33. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1956; 226: 497-509.

34. American Association for Clinical Chemistry Task Force on Cyclosporin Monitoring. Critical issues in Cyclosporin monitoring: Report of the task force on Cyclosporin monitoring. Clin Chem 1987; 33(7): 1269-88.

35. Elia M, Behrens R, Northrop C, Wraight P, Neale G. Evalation of mannitol, lactulose and ^{51}Cr-labelled ethylenediaminetetra-acetate as markers of intestinal permeability in man. Clin Sci 1987; 73: 197-204.

36. Hamilton I, Rothwell J, Archer D, Axon ATR. Permeability of the rat small intestine to carbohydrate probe molecules. Clin Sci 1987; 73: 189-96.

37. Joyce Loebl. Image analysis: Principles and practice. Great Britain: Joyce Loebl, 1985.

38. Erickson RA, Rivera N. Effect of difluoromethylornithine (DFMO) on NSAID-induced intestinal injury in rats. Dig Dis Sci 1992; 37(12): 1833-9.

39. Pahl MV, Erickson RA, Vaziri ND, Khamiseh G, Oveisi F, Jan K, *et al.* Intestinal morphometry and bile acid-induced mucosal injury in chronic experimental renal failure. J Lab Clin Med 1990; 115: 572-8.

40. Field M, Fromm D, McColl I. Ion transport in rabbit ileal mucosa. I, Na and Cl fluxes and short-circuit current. Am J Physiol 1971; 220(5): 1388-96.

41. Fedorak RN, Chang EB, Madara JL, Field M. Intestinal adaptation to diabetes: Altered Na-dependent nutrient absorption in streptozocin-treated chronically diabetic rats. J Clin Invest 1987; 79: 1571-8.

42. P.F. Heeckt, W.M. Halfter, K.K.W. Lee, W.H. Schraut & A.J. Bauer, Prolonged in vivo administration of cyclosporine causes enteric smooth muscle hyperplasia in normal rat intestine and small bowel grafts, in Abstracts of the IIIrd International Symposium on Small Bowel Transplantation, P97, Paris, November, 1993.

43. Wysocki GP, Gretzinger HA, Laupacis A, Ulan RA, Stiller CR. Fibrous hyperplasia of the gingiva: A side effect of cyclosporin A therapy. Oral Surg Med Oral Pathol 1983; 55: 274-8.

44. Kahan BD. Cyclosporine. NEJM; 321(25): 1725-38.

45. Thai BL, Dumont M, Michel A, Erlinger S, Houssin D. Cholestatic effect of Cyclosporine in the rat. Transplantation 1988; 46(4): 510-2.

46. Roman ID, Monte MJ, Gonzalez-Buitrago JM, Esteller A, Jimen R. Inhibition of hepatocytary vesicular transport by Cyclosporin A in the rat: Relationship with cholestasis and hyperbilirubinemia. Hepatology 1990; 12(1): 83-91.

47. Stone B, Warty V, Dindzans V, Van Thiel D. The mechanism of Cyclosporine-induced cholestasis in the rat. Transplant Proc 1988; 20 (suppl 3): 841-4.

48. Stone BG, Udani M, Sanghvi A, Warty V, Plocki K, Bedetti CD, Van Thiel DH. Cyclosporin-A-induced cholestasis: The mechanism in a rat model. Gastroenterol 1987; 93: 344.

49. Bohme M, Buchler M, Muller M, Keppler D. Differential inhibition by cyclosporins of primary-active ATP-dependent transperters in the hepatocyte canalicular membrane. FEBS 1993; 333(1,2): 193.

50. Kadman M, Klunemann C, Bohme M, Ishikawa T, Gorgas K, Otto G, *et al.* Inhibition by cyclosporin A of adenosine triphosphate-dependent transport from the hepatocyte into bile. Gastroenterol 1993; 104: 1507-14.

51. Moseley RH, Johnson TR, Morissette JM. Cyclosporine (CsA) inhibition of taurocholate transport in rat liver plasma membrane vesicles. Gastroenterology 1989; 96: A634.

52. Chan P, Scoble JE, Senior JM, Varghese Z, Sweny P, Moorhead JF. Cyclosporin inhibition of glucose transport in cell culture. ransplant Proc 1989; 21(1): 922-3.

53. Hopfer U. Membrane transport mechanisms for hexoses and amino acids in the small intestine. In: Johnson LR, editor. Physiology of the Gastrointestinal Tract, 2nd ed. New York: Raven Press, 1987: 1499-1526.

54. Philpott DJ, Butzner JD, Meddings JB. Regulation of intestinal glucose transport. Can J Physiol Pharmacol 1992; 70: 1201-7.

55. Madara JL, Loosening tight junctions: Lessons from the intestine. J Clin Invest 1989; 83: 1089-94.

56. Windholz M, Budavari S, Blumetti RF, Otterbein ES, editors. The Merck Index, 10th edition. Rahway, NJ: Merck & Co., Inc., 1983.

57. Peters TJ, Bjarnason I. Uses and abuses of intestinhal permeability measurements. J Gastroenterol 1988; 2(3): 127-32.

58. Shaw LM. Advances in Cyclosporin pharmacology, measurement and therapeutic monitoring. Clin Chem 1989; 35(7): 1299-1308.

59. Shaw LM, Yatscoff RW, Bowers LD, Freeman DJ, Jeffrey JR, Keown PA, *et al.* Canadian concensus meeting on cyclosporin monitoring: Report of the concensus panel. Clin Chem 1990; 36(10): 1841-6.

CHAPTER IV

FK506

INTRODUCTION

Despite some of the clinical successes obtained with CsA, most outcomes of SBT have been limited. As previously discussed, the small bowel is extremely immunocompetent, and it is likely that this strong immunogenicity demands more specific and powerful immunosuppressive regimens. Thus, numerous attempts over the past decade have looked into the development of newer agents for use in SBT.

One of the more studied and clinically applied agents to date is FK506. As discussed earlier, its proposed mechanism of action is similar to CsA, however, it is 10-100 times more potent with respect to suppression of *in vitro* and *in vivo* rejection reactions (1, 2). Because of this similar mechanism of action, it also appears to qualitatively have similar adverse effects as CsA (3, 4).

Hoffman *et al* from Pittsburgh were one of the first to demonstrate the ability of FK506 to prolong survival of small intestinal allografts between strongly histoincompatible rats; strain combinations within which even prolonged courses of CsA could not prevent acute rejection (5). Using a dose of 2 mg/kg/d for 6 days then 1 mg/kg on alternate days for a further 24 days, a mean survival time (MST) of 51 days was achieved with no cases of acute rejection or graft-versus-host-disease (GVHD). However, 50% of the rats eventually succumbed to chronic rejection. No toxic effects of FK506 were noted. Further studies by this group using the same dose, but for only 4 days (6), showed long-term (>180 days) functional graft survival in 5 of 5 fully allogeneic transplanted with no signs of rejection or GVHD, but these were of less histoincompatibility; one rat eventually died from pneumonia.

With experience, the toxicity of FK506 was becoming realized, and lower dosage schedules developed. Hatazawa *et al* were able to obtain long-term survival (>8 weeks) in fully allogeneic

transplanted rats with doses of 1 mg/kg/d for 8 weeks (7). No signs of rejection or GVHD were noted. A much lower dosage schedule developed by the Pittsburgh group, 0.32 mg/kg/d for 13 days, significantly prolonged allograft survival (>175 days) in 80% of rats transplanted across minor histoincompatibility strains and allowed for induction of donor-specific unresponsiveness (DSU) as tested by skin grafting after SBT (8). Across major histoincompatible combinations, however, allograft rejection could only be delayed, but not prevented (MST of 38 days compared to 6.4 days in controls) and DSU could not be induced. Others have had similar results with doses ranging from 0.1 to 0.5 mg/kg/d (9).

With clinical successes in transplantation of other organs and some successes in animal models of SBT, FK506 has been used by several centers for immunosuppression of SBT alone, in combination with the liver, or as part of a multivisceral graft (10, 11). However, adequate nutritional function is also imperative to the success of these allografts. Aside from known minor gastrointestinal side effects ranging from minor cramps and abdominal distention to nausea, vomiting and diarrhea (12), little work has been published describing the absorptive capacity of the small bowel in these animal and clinical transplants on FK506. As CsA has been found to alter some aspects of nutrition and small bowel function, and since the spectrum of side effects of FK506 appear to be similar to that of CsA, we wished to investigate what effects FK506 had, if any, on growth, nutrition and small bowel function in a manner similar to our studies of CsA.

MATERIALS AND METHODS

ANIMALS

Juvenile male Lewis rats (275-300 g) were obtained from Charles River Canada, St. Constant, P.Q., and housed in individual plexiglass cages with free access to water and standard lab rat chow (Lab Diet, PMI Feeds, St. Louis, MO.) with an approximate composition of 23.0% protein, 4.5% fat, 6.0% fiber, 8.0% ash and 2.5% minerals. Day/night cycles were 12 hours and the temperature was maintained at

20 ± 2°C. Feed intake was monitored weekly, and body weight changes, biweekly. Animal care was in accordance with the guidelines of the Canadian Council of Animal Welfare (13). The experimental protocol was approved by the Animal Welfare Committee of the University of Alberta.

EXPERIMENTAL GROUPS

The FK506-treated animals (n=10) received an immunosuppression protocol equivalent to that described above, which is able to prolong allograft survival across major histoincompatible strains of rats. FK506, 1 mg/kg, suspended in carboxymethylcellulose, was injected subcutaneously (s.c.) into the nape of the neck qd for 6 days, followed by 2 mg/kg on alternate days for 5 more weeks. Control animals received a corresponding volume/kg of the drug vehicle, on the same schedule. As discussed in Chapter I of this thesis, the complete study undertaken was performed in two sets of experiments. This subsequently provided two control groups against which the test group could be compared including "control 2" (n=10) which was studied alongside FK506 in the second set of experiments, and an overall control group of n=20 consisting of "control 2" and "control 1" (n=10), which was utilized in the first set of experiments. Each control group was divided in half, with 5 animals received carboxymethylcellulose (CMC), and 5 receiving MCT oil, the vehicle used with the CsA studies.

DRUGS

FK506, a generous gift of Fujisawa USA, Deerfield, IL, was dissolved in one part normal saline and then further diluted with 9 parts CMC, to a final concentration of 4.8 mg/ml for the first 6 days and they 2.4 mg/ml, thereafter. It was freshly prepared every week and stored in the dark at 4°C. Drug concentrations were prepared so that equivalent volumes (0.83 ml/kg) were injected throughout the study. Control animal received equivalent volumes per kg of the CMC vehicle.

***IN VIVO* NUTRIENT BALANCE STUDIES**

After 33 days of treatment, the animals were placed in individual metabolic cages, preconditioned for 3 days, and then underwent a 3 day balance study with daily measurement of food-intake and fecal collection. Standard lab rat chow was given *ad lib*, with a composition as described above. Total

carbohydrate and fat content of feed and feces were determined using standard methods (14, 15). Briefly, feed and collected feces were completely desiccated in a freeze-dryer, and the percentage water determined directly by the change in total weight. Dried feces were then ground into a powder, and 0.2 g of sample used to determine the total energy content using a Gallenkamp Ballistic Bomb Calorimeter (London, England). O.25 g of sample was then rehydrogenated and the fat extracted into the solvent layer of a 0.9% sodium-chloride-chloroform:methanol (2:1) extraction mixture. After a second extraction of the aqueous layer, to ensure completeness, the solvent was evaporated off and the final contents weighed to determine the amount of total fat extracted. From these results, the percentages of energy and fat absorption from feed intake were calculated directly.

HEMATOLOGICAL PARAMETERS

Complete blood counts were determined on each animal at sacrifice using a M430 Coulter Counter. Electrolytes, creatinine and blood glucose levels were also determined using a Beckman Astra-Eight analyzer.

DRUG LEVELS

FK506 whole blood levels were drawn at sacrifice. Peak levels (drawn two to six hours post-injection) and trough levels (drawn 24 hours post-injection) were determined by micro particle enzyme immunoassay (IM_x®, Abbot Diagnostics, Chicago, IL).

HYPERREACTIVITY STUDIES

Subjective observations of increased aggression in the test rats was quantitated and compared with controls using a modified version of a reactivity test for rats as described by Albert and Richmond (16). Briefly, the animals' response to 6 test stimuli were rated on a scale of 0 to 3, with 0 indicating no response and 3 indicating attack or highly aggressive response (vocalization and/or biting). Test items were: (1) Presentation of a pencil just in front of the rat's snout; (2) A sharp tap on the back with a pencil; (3) Presentation of a gloved hand before the snout; (4) Gentle prods on the side of the rat's body with a

blunt one inch diameter stick; (5) Attempted capture by the tail; and (6) Attempted grasping around the body. The total score was recorded, with a maximum reactivity score possible of 18.

IN VIVO INTESTINAL PERMEABILITY

One day following the balance studies, animals were fasted for 6 hours and then gavaged with 200 μCi of ^{99}Tc-DTPA in 1.5 mlo of water. Urine was quantitatively collected over 20 hours while the animals were allowed *ad lib* access to food and water. The percent urinary recovery of the labeled probe was determined by simultaneously counting aliquots of the original test solution and urines in a γ-counter, both having undergone the same period of radioactive decay.

On the following day, animals were fasted overnight and then gavaged with 100 mg of mannitol and 100 mg of lactulose in 2 ml of water during which time the animals were allowed free access to water but not food. Urine was quantitatively collected and urinary recovery of each compound was measured using high performance liquid chromatography and reported as both the percentage recovery of the total oral dose of each carbohydrate probe, and the ratio of lactulose to mannitol recovered. This latter measurement is felt to best reflect the permeability of the bowel per unit surface area (17, 18).

VILLUS MORPHOMETRY AND DENSITY

At sacrifice, sections of jejunum and ileum from control and test animals were fixed in formalin and mounted in paraffin blocks. They were cut so that both longitudinal and cross-sections of bowel were obtained which were then stained with haematoxylin and eosin. As an indirect measurement of mucosal surface characteristics, sections of ileum and jejunum, for each group, were photomicrographed at a magnification of 100X. Morphometric analyses were performed using an Image analysis system (Joyce Loebl, Magiscan) (19). Image analysis systems have been used to efficiently determine morphometric parameters of large numbers of intestinal villi in several recent studies analyzing intestinal mucosal architecture (20,21). Parameters studied included villus length, as measured from the lamina propria, width and overall sagittal section area for 25 villi per histological section. Three sections from both the jejunum and ileum for each test group and four sections from both the jejunum and ileum of the controls

were analyzed, resulting in 75 to 100 villi being measured per group for each region of small bowel. The number of villi per microscopic field (at 20X magnification) were determined as a measure of villus density.

IN VITRO GLUCOSE FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

After 6 weeks of treatment, animals were sacrificed with an intraperitoneal pentobarbital overdose and *in vitro* small bowel function studies were performed. Segments of distal ileum and proximal jejunum, taken approximately 2 to 3 cm from the ileocecal valve and ligament of Trietz, respectively, were quickly excised, rinsed with ice-cold normal Ringers solution, and stored in ice-cold Ringers gassed with carbogen (5% CO_2 in O_2) during the preparation for mounting onto Ussing chambers as previously described (22,23). Briefly, the intestine was split along the mesenteric border and segments of approximately 2 cm were then quickly stripped of the serosa and underlying muscle layers and then clamped into Ussing chambers . This subsequently divides the chamber into mucosal and serosal compartments. Tissues were bathed on both sides by normal Ringers (Na^+, 143 mM; K^+, 5 mM; Ca^{2+}, 1.25 mM; Mg^{2+} 1.1 mM; Cl^-, 123.7 mM; HCO_3, 25 mM; $HPO_4 + H_2PO_4$, 1.95 mM) with 20 mM fructose, and gassed with carbogen to pH of 7.4. A circulating water bath maintained the chamber temperature at 37°C. For measurement of unidirectional fluxes, 3-O-methyl-D-glucose (3OMeG) was present at a concentration of 20 mM on both mucosal and serosal sides and 5 μCi of [^{3}H]-3OMeG was added to either the mucosal or serosal side of the tissue. To ensure steady-state conditions, tissues were maintained under these conditions for 20 minutes. Previous studies have shown that glucose flux is constant over this time period (23). Unidirectional mucosal-to-serosal (J_{ms}) and serosal-to-mucosal (J_{sm}) fluxes were measured in paired tissues by collection of aliquots of incubation solution from each side of the chambers and determining the radioactivity in a Beckman LS5801 β-Scintillation Counter (California). Tissue pairs were discarded if electrical conductance (see below) varied by >15%. Four consecutive 5 minute fluxes were determined. Tissue response to 5 mM theophylline was used to confirm viability at the completion of the experiment.

Electrophysiological parameters were monitored throughout. The spontaneous transepithelial electrical potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (I_{sc}) with an automatic voltage clamp (DCV 1000 World Precision Instruments, New Haven, CT), except for 3-5 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue conductance (G) was calculated from potential difference and I_{sc} according to Ohm's law, where voltage (V) = current (I_{sc}) x resistance (R) and conductance (G) is the reciprocal of R (22).

ENTEROCYTE METABOLIC FUNCTION

At sacrifice, enterocytes were isolated from the distal jejunum, tested for viability and analyzed with respect to mitochondrial substrate utilization and energy production as described elsewhere (24). Briefly, after isolation of the cells, aliquots were incubated for 60 minutes in the presence of either [^{14}C]glucose or 2-[^{14}C]pyruvate and the amount of each substrate utilized quantitated by the amount of [^{14}]CO_2 released (25). Lactate and pyruvate production was then determined from aliquots of cells over a 60 minute incubation period and reported as mg/h/mg protein. Finally, intracellular ATP levels were quantitated after extraction from cell suspensions, using the luciferase/Luciferin assay kit (Sigma Diagnostics) in a Lumat lumnometer.

STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using a computerized statistical package (SigmaStat Statistical Analysis System, Jandel Corporation). Means between the two groups (controls vs treatment) were compared using the Student's t-test unless the distribution was nonparametric (as determined directly by the program, using the Kolmogorov-Smirnov test of normality, with a p-value of >0.05) in which case a distribution-free test comparing the medians is more appropriate, and was performed with a Mann-Whitney rank sum test. A p-value of <0.05 was considered statistically significant. No statistically significant differences were determined between the

MCT and CMC treatments, and in all but three sets of results (stool water content, lactulose/mannitol permeability and villus morphometry), no differences were found between the two control groups ("control 1" and "control 2"). Subsequently, all control animals were combined into one control group with n=20. In studies where the two significantly differed , the differences were assumed to be secondary to altered laboratory processing of the samples, and the FK506 group was compared to its respective "control 2" group, only.

RESULTS

WEIGHT GAIN, FEED INTAKE & *IN VIVO* NUTRITIONAL BALANCE STUDIES

The rate of weight gain over the six week period was significantly reduced, by 79%, in those animals receiving FK506 as compared to controls ($p<0.001$) despite a significant 9% increase in feed intake ($p=0.002$). These effects are tabulated in Table IV-1.

IN VIVO NUTRITIONAL BALANCE STUDIES

Energy absorption (kcal) was significantly reduced in the FK506-treated animals by 14.5% compared to controls, and fat absorption decreased by 20% ($p<0.001$ for both values) (Table IV-1).

OTHER GASTROINTESTINAL EFFECTS

The treated animals developed significant diarrhea, as manifested by a 113% increase in stool water content compared to controls ($p<0.001$).

SERUM & HEMATOLOGICAL PARAMETERS

Serum K^+ was increased by 32% and serum creatinine by almost two-fold. Neither of these results reached statistical significance, however, because of large standard errors. The mean random serum glucose was comparable to controls. The animals developed significant anemia and leukopenia, with RBC, hemoglobin and hematocrit significantly reduced by 9-13% compared to controls, and WBC by 32%. These results are presented in Table IV-2.

DRUG LEVELS

At sacrifice, the peak and trough levels were 39.08 ± 8.83 ng/ml and 24.68 ± 3.10 ng/ml, respectively.

HYPERREACTIVITY TESTING

Marked aggressive behavior and hyperreactivity was observed in the FK506 animals within 1 week of beginning the drug injections. This appeared to peak at about 2 or 3 weeks (subjective observation), and then slowly began to abate. Testing at 5 weeks revealed a significantly increased hyperreactivity score (mean score of 6.2 ± 0.7) compared to controls (mean score of 3.0 ± 0.3, $p<0.05$).

IN VIVO BOWEL PERMEABILITY

Percent recovery of ^{99}Tc-DTPA was significantly increased by 94% in the FK506 group compared to controls (Table IV-3). Permeability to both lactulose and mannitol was increased by over two-fold. Surprisingly, however, the lactulose/mannitol ratio was significantly reduced by 16% compared to control animals.

VILLUS MORPHOMETRY AND DENSITY

FK506 induced no significant changes in villus morphometry or density throughout the small bowel compared to controls (Table IV-4).

***IN VITRO* 3OMeG FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS**

The effects of FK506 on *in vitro* 3OMeG transmural fluxes are summarized in Table IV-5. In the jejunum, J_{sm} was significantly increased by 90%, with a 92% increase in J_{ms}. This resulted in a 80% increase in J_{net}. In the ileum, an almost three-fold (164%) increase in J_{ms} was insufficient to compensate for the four-fold (278%) increase in J_{sm}. Subsequently, there was a loss of all net uptake, reflected in a significant 39% reduction in I_{sc}. Large increases in electrical conductance, by 89% in the jejunum and 260% in the ileum corresponded to these changes in J_{sm} throughout the bowel.

ENTEROCYTE METABOLIC FUNCTION

Enterocytes isolated from animals treated with FK506 produced 54% less CO_2 when incubated in the presence of 20 mM D-glucose as compared with enterocytes isolated from control animals. The release of $^{14}CO_2$ from cells incubated in the presence of [^{14}C]-pyruvate was used as a measure of mitochondrial function. Cells isolated from the FK506-treated animals demontrated a 50% decrease in CO_2 production compared to the control animals. There was no difference in lactate or pyruvate production, however. Finally, mean ATP levels were decreased by 33% in the FK506 group relative to controls. These differences were all statistically significant. Taken together, this data indicates a reduced mitochondrial respiratory capacity in the enterocytes of the FK506-treated animals. These results are tabulated in Table IV-6.

DISCUSSION

Significant effects on animal growth, well-being, nutrient absorption and *in vitro* small bowel and enterocyte function were induced by treatment with FK506. It must be noted that the dose utilized, 2 mg/kg on alternate days, is relatively high compared to current FK506 regimens in both animal and clinical studies. However, in the rat model, it appears to be the dose required to permit highly immunogenic allograft acceptance between major histoincompatible rat strains (i.e. ACI →Lewis) (5,8). Similar high doses have also been required for other "difficult-to-transplant" organs such as renal subcapsular pancreatic islet allografts (26).

Indeed, over the past two years, because of the discovery of numerous toxicities of this agent, dosages have been significantly reduced, and it is felt that careful monitoring of FK506 plasma levels is necessary to reduce these toxic effects (4,27-29). However, some adverse effects such as minor neurological disturbances, hyperkalemia and hypertension may not be as dependent on serum trough levels (4,30). Also, despite maintenance of FK506 plasma levels between the recommended 0.2 and 5.0 ng/ml (30), side effects including nephrotoxicity, hyperkalemia, hypertension, seizures, tremors, glucose intolerance and

gastrointestinal disturbances are frequent (31). In our studies, serum trough levels were 5 to 10 times higher than these recommendations.

Significant toxicity was noted in the treated animals, consisting of increased creatinine and K^+ levels, anemia, leukopenia, neurotoxicity (manifested as hyperreactivity and aggression) and diarrhea. However, as noted above, many of these adverse effects can occur despite appropriate serum trough levels, and are not necessarily dependent on the dose utilized.

Weight gain was significantly inhibited in animals receiving this agent, despite a significant 9% increase in feed intake. This has also been described by others in rats treated with 3 mg/kg/d, s.c., for 35 days (32), and 4 mg/kg/d. p.o. for 34 days (33). Similar effects have been described in monkeys treated with 1 mg/kg/d (27). The chronic administration of this agent likely plays a role in that short-term (4 day) FK506 therapy of comparable dosage was found to have no effect on growth or nutritional status in a rat transplant model (6).

This adverse weight gain can only be partially accounted for by the 20% and 14.5% decreases in fat and energy absorption observed, respectively. Based on calculations of energy available for growth after basal metabolic requirements are considered, this is reduced by only 16.5% relative to controls; this is proportionally far less than the marked decrease in weight gain (34). These calculations are outlined in Appendix III and discussed further in chapter VIII

As villus morphometry and density appears to be maintained in these animals, decreased nutrient absorption is unlikely to be secondary to any decrease in mucosal absorptive area. Like CsA, fat malabsorption secondary to FK506 may be the result of induced cholestasis (35-37). Indeed, FK506 appears to have similar effect as CsA on bile flow in dogs, inducing a chloride-rich choleresis (38, 39). More recently, however, a cholestatic effect of FK506 has come under scrutiny, with the finding that inhibition of ATP-dependent bile salt transport, the proposed mechanism of CsA-induced cholestasis, requires FK506 concentrations which are similar to CsA concentrations (40). However, because of the known 10-100 times increased potency of FK506 and therefore, lower doses used, it is unlikely that this

mechanism is in play in animal models and clinical situations. [A 50% inhibition in rats requires FK506 concentrations in the range of 7 to 8 μg/ml which are 1000-fold higher that recommended serum levels of the drug (40)].

Decreased energy absorption in these animals is likely multifactorial. As will be discussed below, *in vitro* net uptake of 3OMeG was obliterated in the ileum of these animals. Although the jejunum is normally responsible for the majority of carbohydrate absorption, we also observed significant changes in 3OMeG fluxes in this region of the bowel. Also, it may be possible that other aspects of carbohydrate digestion, not specifically tested in these studies (i.e. enyzymatic hydrolysis of dissacharides), may be affected in these animals. Alternatively, it is possible that some degree of protein malabsorption may account for some of the caloric loss in the stools. Although we did not look directly at protein absorption, and indeed, have found previously that CsA has little effect on protein absorption (41), others have found that CsA can produce a reversible reduction in neutral amino acid absorption during perfusion experiments in intestinal autografts (42). This could potentially also occur with long-term FK506 treatment.

Looking directly at the *in vitro* glucose fluxes (Figures 3A and 3B and Tables 5A and 5B), the control animals demonstrated unidirectional and net glucose fluxes which were similar to those previously described in normal Lewis strain rats (23), confirming that chronic injection of the vehicles used did not have any significant effects on the flux or electrophysiological parameters studied. Treatment with FK506, however, induced significant increases in unidirectional flux in both the mucosal-to-serosal and serosal-to-mucosal directions, throughout the bowel. It was most significant, however, in the ileum. These effects are similar to that seen in the ileum with long-term treatment with CsA (discussed in the preceding chapter), however, occur to a much larger degree. The reasons for these changes are still not understood, but may be secondary to alterations in intestinal permeability (43, 44). The proportionally increased conductance in both the jejunum and ileum would support this concept, whereby an increase in back diffusion of glucose from the serosal to mucosal sides of the bowel mucosa may result in a relative

increase of glucose present at the brush border membrane. This, in turn, may stimulate a second messenger system to induce upregulation of active glucose transport and/or increase paracellular movement of glucose to the serosal side., resulting in enhanced glucose absorption (45). This would occur at a large energy expense to the enterocyte, potentially having a negative effect on other nutrient absorption or processing duties.

The effects of FK506 on intestinal permeability in these and other studies are mixed. The 2 to 3-fold increases in electrical conductance, *in vitro* indicate that permeability is largely increased. *In vivo*, 2-fold increases in ^{99}Tc-DTPA, lactulose and mannitol recoveries were also observed. However, it is possible that these increases may reflect a profound increase in gut motility, allowing longer exposure time of the probes to the transcellular pores and intracellular tight junctions, rather than an absolute increase in intestinal permeability. Indeed, the lack of an increase in the lactulose/mannitol ratio suggests this. Unfortunately, as explained in the previous chapter, *in vivo* permeability studies may be fraught with confounding variables of variations in intestinal transit, mucosal water flux, blood and lymphatic flow, renal function and excretion and tissue or luminal metabolism of the probes (46).

Many of the effects seen may be related, however, to the profound effects on mitochondrial energy metabolism observed. Studies investigating the effects of FK506 on mitochondrial metabolism are scarce. FK506 has been shown to induce significant mitochondrial swelling in beta cells of pancreatic isografts (32). In addition, recent indirect evidence of altered mitochondrial function has been suggested by Krentz *et al* (47). They found a significant increase in the of serum ratio of 3-hydroxybutyrate:acetoacetate in clinically stable liver transplant recipients on long-term FK506 or CsA, relative to healthy control subjects. Since the circulating concentration of acetoacetate is dependent on the intramitochondrial concentration ratio of NAD+:NADH via the 3-hydroxybutyrate dehydrogenase reaction (48, 49), this increased ratio implies a more reduced hepatic intramitochondrial redox state in these patients, which may represent a subtle defect of mitochondrial function in the hepatic allografts. This could potentially be the result of the immunosuppression as the authors observed significantly low fasting blood lactate levels

in these patients, as compared to hyperlactatemia seen in chronic liver disease, arguing against dysfunction of the hepatic grafts as the cause of the altered ketone metabolism. Furthermore, Henke *et al* have found that FK506 (and CsA) can induce respiratory dysfunction in renal mitochondria respiration in the rat and inhibit net uptake of ATP (50). However, unlike CsA, this occurs at *in vitro* concentrations which are significantly higher than FK506 concentrations found *in vivo*.

One can speculate as to the widespread effects these alterations in energy metabolism may have on growth and small bowel function. Energy is required for all cellular processes, including protein synthesis. Thus, any energy deficit may negatively affect these processes and inhibit the overall maintenance of body cell mass as well as cells with rapid turnover, such as the hematopoietic system. This may also account for the possible increase in bowel permeability, as the functional integrity of intraepithelial tight junctions appears to be strongly dependent on energy (51).

In summary, FK506, at the dose used, has significant adverse effects on growth and animal well-being in the normal rat. Nutrient absorption is significantly compromised with respect to fat and energy, however these changes are unlikely to entirely account for the severely impaired weight gain observed. At the small bowel level, significant energy wastage may be occurring in an attempt to maintain adequate nutrient uptake while compensating for a profound increase in intestinal permeability. Finally, alterations in mitochondrial energy production may have more widespread effects on the animals than at the enterocyte level alone. Although these studies need to be repeated utilizing lower doses of FK506, they indicate the potential for small bowel dysfunction, malabsorption and impaired growth with FK506, which may become especially relevant when administered to patients with already borderline nutritional status, such as small bowel transplant recipients.

Table IV-1

Effects of FK506 on Weight gain, Feed intake and Energy and Fat Absorption

	Weight Gain (g/d)	Feed Intake (g/kg/d)	% Energy Absorption	% Fat Absorption
Control (n=20)	3.15 ± 0.07	69.42 ± 1.11	81.3 ± 0.6	76.1 ± 0.9
FK506 (n=10)	0.65 ± 0.14 ***79%↓** $p < 0.001$	76.00 ± 1.68 ***9%↑** $p = 0.002$	69.5 ± 1.0 ***14%↓** $p < 0.001$	61.0 ± 1.0 ***20%↓** $p < 0.001$

Values are means ± SEM

***** significant difference compared to controls ($p<0.05$); compared with student's t-test

% values indicated relative to controls (↑, increase; ↓, decrease).

Table IV-2

Effects of FK506 on Serum Electrolyte and Hematological Values

	Na^+ (mmol/l)	K^+ (mmol/l)	Creatinine (mmol/l)	Glucose (mg/dl)	WBC (10^3c/mm^3)	RBC (10^3c/mm^3)	HgB (g%)	Hct (%)
Control 2 (n=10)	143.2 ± 0.6	7.6 ± 0.8	0.42 ± 0.04	128.5 ± 0.5	7.8 ± 0.6	8.6 ± 0.2	16.4 ± 0.2	43.8 ± 0.9
FK506 (n=9)	138.8 ± 1.7 3%↓ p>0.1	10.1 ± 2.1 32%↑ p>0.1	0.81 ± 0.15 93%↑ p 0.1	113.6 ± 12.8 12%↓ p>0.43	5.3 ± 1.0 ***32%↓** p=0.03	7.5 ± 0.4 ***13%↓** p=0.02	14.9 ± 0.6 *** 9%↓** p 0.01	38.6 ± 1.4 ***12%↓** p 0.00

Values are means ± SEM

* significant difference compared to controls ($p<0.05$); compared with student's t-test

% values indicated relative to controls (↑, increase; ↓, decrease)

WBC, white blood cell count; RBC, red blood cell count; HgB, hemaglobin; Hct, hematocrit

Table IV-3

Effects of FK506 on *In Vivo* Intestinal Permeability

	^{99}Tc-DTPA	Mannitol	Lactulose	Lactulose/ Mannitol
Control 2 (n=10)	2.24 ± 0.18	1.97 ± 0.11	0.81 ± 0.04	0.42 ± 0.06
FK506 (n=10)	4.35 ± 0.90 ***94%↑** p=0.009	4.49 ± 0.60 ***128%↑** p<0.001	1.63 ± 0.27 ***100%↑** p<0.001	0.35 ± 0.02 ***16%↓** p=0.020

Data is presented as percent recovery of orally administered marker in urine; mean ± SEM

***** significant difference compared to controls ($p<0.05$); compared with student's t-test

% values indicated relative to controls (↑, increase; ↓, decrease).

Table IV-4
Effects of FK506 on Villus Morphometry and Density

	Width ($\mu m \times 10^2$)	Height ($\mu m \times 10^2$)	Area ($\mu m^2 \times 10^2$)	# Villi per 20X field
JEJUNUM				
Control 2 (n=10)	2.00 ± 0.05	6.86 ± 0.07	8.03 ± 0.21	5.41 ± 0.19
FK506 (n=9)	1.90 ± 0.06 5%↓ p=0.25	6.80 ± 0.08 1%↓ p=0.54	7.82 ± 0.24 3%↓ p=0.47	5.63 ± 0.34 4%↑ p=0.573
ILEUM				
Control 2 (n=10)	2.06 ± 0.06	4.07 ± 0.07	4.94 ± 0.17	4.65 ± 0.33
FK506 (n=9)	2.15 ± 0.10 4%↑ p=0.595	1.13 ± 0.07 1%↑ p=0.26	5.24 ± 0.27 6%↑ p=0.851	5.31 ± 0.20 14%↑ p=0.188

Data is presented as villus width, height from lamina propria and saggital section area; expressed as mean ± SEM.
* significant difference compared to controls ($p<0.05$); compared with student's t-test.
% values indicated relative to controls (↑, increase; ↓, decrease).

Table IV-5
Effects of FK506 on *In Vitro* 3OMeG Fluxes and Electrophysiological Parameters

	J_{ms}	J_{sm}	J_{net}	PD	I_{sc}	G
JEJUNUM						
Control (n=20)	1.24 ± 0.08	0.84 ± 0.06	0.41 ± 0.05	1.70 ± 0.12	39.29 ± 3.16	22.56 ± 1.54
FK506 (n=9)	2.35 ± 0.14 ***90%↑** p<0.001	1.61 ± 0.14 ***92%↑** p<0.001	0.74 ± 0.09 ***80%↑** p<0.001	1.31 ± 0.17 23%↓ p=0.059	32.78 ± 3.91 17%↓ p=0.118	42.73 ± 3.44 ***89%↑** p<0.001
ILEUM						
Control (n=20)	1.19 ± 0.08	0.82 ± 0.08	0.36 ± 0.06	2.26 ± 0.15	40.86 ± 2.59	18.25 ± 1.50
FK506 (n=9)	3.14 ± 0.45 ***164%↑** p<0.001	3.10 ± 0.54 ***278%↑** p<0.001	0.04 ± 0.16 89%↓ p=0.194	0.38 ± 0.11 ***83%↓** p=0.003	24.77 ± 4.03 ***39%↓** p<0.001	65.63 ± 10.71 ***260%↑** p<0.001

Values are means ± SEM. J_{ms}, flux from mucosa to serosa; J_{sm}, flux from serosa to mucosa; J_{net}, numerical difference between J_{ms} and J_{sm} in μmol/cm²/hr; PD, potential difference in mV; Isc, short-circuit current in μAmp/cm²/hr; G, conductance in mS/cm².

*significant difference compared to controls ($p<0.05$); compared with student's t-test; % values indicated relative to controls (↑, increase; ↓, decrease).

Table IV-6

Effects of FK506 on *In Vitro* Mitochondrial Energy Production

	[^{14}C]Glucose utilization (ng/hr/mg protein)	[^{14}C]Pyruvate utilization (nmol/hr/mg)	Lactate production (mg/hr/mg protein)	Pyruvate production (mg/hr/mg protein)	ATP production (nmol/μg protein)
Control (n=4)	24.20 ± 1.21	1.19 ± 0.15	0.10 ± 0.02	0.0067 ± 0.0006	1.32 ± 0.12
FK506 (n=4)	11.11 ± 3.42 ***45%↓** p=0.005	0.60 ± 0.11 ***50%↓** p=0.007	0.10 ± 0.02 0%↔ p=0.89	0.0098 ± 0.0038 12%↑ p=0.50	0.89 ± 0.04 ***33%↓** p=0.015

Values are means ± SEM

* significant difference compared to controls ($p<0.05$); compared with student's t-test

% values indicated relative to controls (↑, increase; ↓, decrease).

REFERENCES

1. Sadawa S, Suzuki G, Kawase Y, Takaku F. Novel immunosuppressive agent FK506: in vitro effects on the cloned T-cell activation. J Immunol 1987; 139: 1797.

2. Goto T, Kino T, Hatanaka H, Okuhara M, Kohsaka M, Aoki H, Imanaka H. FK506: Historical perspectives. Transplant Proc 1991; 23(6): 2713-7.

3. Fung JJ, Alessiani M, Abu-Elmagd K, Todo S, Shapiro R, Tzakis A, *et al.* Adverse effects associated with the use of FK506. Transplant Proc 1991; 23(6): 3105-8.

4. Wallemacq PE, Reding R. FK506 (Tacrolimus), a novel immunosuppressant in organ transplantation: Clinical, biomedical, and analytical aspects. Clin Chem 1993; 39(11): 2219-28.

5. Hoffman AL, Makowka L, Banner B, Cai X, Cramer DV, Pascualone A, Todo S, Starzl TE. The use of FK506 for small intestine allotransplantation: Inhibition of acute rejection and prevention of fatal graft-versus-host disease. Transplantation 1990; 49(3): 483-90.

6. Lee KKW, Stangl MJ, Todo S, Langrehe JM, Starzl TE, Schraut WH. Successful orthotopic small bowel transplantation with short-term FK506 immunosuppressive therapy. Transplant Proc 1990; 22(1)(suppl 1): 78-9.

7. Hatazawa C, Yamaguchi M, Kato T, Koyama K. Effect of FK506 on bowel transplantation in rats. Transplant Proc 1992; 24(3): 1177.

8. Date K, Okajima K, Takeda Y, Isozaki H, Tezuka K, Ryo T. Effect of FK506 on graft survival in rat small intestinal allografts. Transplant Proc 1992; 24(3): 1173.

9. Utsunomiya H, Tanaka K, Uemoto S, Kato H, Nishizawa T, Yamamoto E, Ozawa K. Effect of FK506 on orthotopic small bowel transplantation in rats. Transplant Proc 1992; 24(3): 1191.

10. Todo S, Tzakis A, Reyes J, Abu-Elmagd K, Casavilla A, Fung JJ, Starzl TE. Intestinal transplantation in humans under FK506. Transplant Proc 1993; 25(1): 1198-9.

11. S. Todo S, Tzakis A, Reyes J, Abu-Elmagd K, Furukawa H, Nour B, *et al.* Clinical intestinal transplantation: three-year experience. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation 1993 Nov; Paris: O2.

12. Japanese FK506 Study Group. Japanese study of FK506 on kidney transplantation: Results of late phase II study. Transplant Proc 1993; 25(1): 649-54.

13. Canadian Council on Animal Care. Guide to the care and use of Experimental Animals. The Council, 1980.

14. Association of Official Analytical Chemist (1986): Methods of analysis (23 Ed.). AOAC, Washington, D.C.

15. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1956; 226: 497-509.

16. Albert DJ, Richmond SE. Septal hyperreactivity: A comparison of lesions within and adjacent to the septum. Physiol & Behav 1975; 15: 339-47.

17. Elia M, Behrens R, Northrop C, Wraight P, Neale G. Evalation of mannitol, lactulose and ^{51}Cr-labelled ethylenediaminetetra-acetate as markers of intestinal permeability in man. Clin Sci 1987; 73: 197-204.

18. Hamilton I, Rothwell J, Archer D, Axon ATR. Permeability of the rat small intestine to carbohydrate probe molecules. Clin Sci 1987; 73: 189-96.

19. Joyce Loebl. Image analysis: Principles and practice. Great Britain: Joyce Loebl, 1985.

20. Erickson RA, Rivera N. Effect of difluoromethylornithine (DFMO) on NSAID-induced intestinal injury in rats. Dig Dis Sci 1992; 37(12): 1833-9.

21. Pahl MV, Erickson RA, Vaziri ND, Khamiseh G, Oveisi F, Jan K, *et al.* Intestinal morphometry and bile acid-induced mucosal injury in chronic experimental renal failure. J Lab Clin Med 1990; 115: 572-8.

22. Field M, Fromm D, McColl I. Ion transport in rabbit ileal mucosa. I, Na and Cl fluxes and short-circuit current. Am J Physiol 1971; 220(5): 1388-96.

23. Fedorak RN, Chang EB, Madara JL, Field M. Intestinal adaptation to diabetes: Altered Na-dependent nutrient absorption in streptozocin-treated chronically diabetic rats. J Clin Invest 1987; 79: 1571-8.

24. Madsen KL, Yanchar NL, Fedorak RN, Sigalet DL. Effect of FK506 and Deoxyspergualin on intestinal metabolic function. submitted.

25. Roediger WE, Truelove SC. Method of preparing isolated colonic epithelial cells (colonocytes) for metabolic studies. Gut 1979; 20: 484-8.

26. Ryu S, Yasunami Y. Necessity of differential immunosuppression for prevention of immune rejection by FK506 in rat islet allografts transplanted intothe liver or beneath the kidney capsule. Transplantation 1991; 52: 599-605.

27. Wijnen RMH, Ericzon BG, Tiebosch ATGM, Beysens AJ, Groth CG, Kootstra G. Toxicity of FK506 in the cynomolgus monkey: Noncorrelation with FK506 serum levels. Transplant Proc 1991; 23(6): 3101-4.

28. McMaster P, Buist L. FK506 in Transplantation. Transplant Proc 1993; 25(4): 2684-5.

29. Backman L, Nicar M, Levy M, Distant D, Eisenstein C, Renard T, Goldstein R, Husberg B, Gonwa TA, Klintmalm G. FK506 trough levels in whole blood and plasma in liver transplant recipients: Correlation with clinical events and side effects. Tansplantation 1994; 57(4): 519-25.

30. Alessiani M, Cillo U, Fung JJ, Irish W, Abu-Elmagd K, Jain A, *et al.* Adverse effects of FK506 overdosage after liver transplantation. Transplant Proc 1993; 25(1): 628-34.

31. Rosh JR, Schwersenz AH, Schwartz M, Moran V, Leleiko N, LeLeiko VMN, Miller CM. Therapy with FK506 in pediatric liver recipients. TransplANT Proc 1994; 26(1): 152-3.

32. Ueki M, YasunamiY, Ina K, Ryu S, Funakoshi A, Kamei T, Ikeda S. Diabetogenic effects of FK506 on renal subcapsular islet isografts in rats. Diabetes Res Clin Pract 1993; 20(1): 11-19.

33. Nalesnik MA, Todo S, Murase N, et al. Toxicology of FK506 in the Lewis rat. Transplant Proc 1987; 29: 89-92.

34. National Academy of Sciences. Nutrient requirements of laboratory animals. Washington, D.C., 1978.

35. Thai BL, Dumont M, Michel A, Erlinger S, Houssin D. Cholestatic effect of Cyclosporine in the rat. Transplantation 1988; 46(4): 510-2.

36. Roman ID, Monte MJ, Gonzalez-Buitrago JM, Esteller A, Jimen R. Inhibition of hepatocytary vesicular transport by Cyclosporin A in the rat: Relationship with cholestasis and hyperbilirubinemia. Hepatology 1990; 12(1): 83-91.

37. Stone B, Warty V, Dindzans V, Van Thiel D. The mechanism of Cyclosporine-induced cholestasis in the rat. Transplant Proc 1988; 20 (suppl 3): 841-4.

38. Reese JC, Fairchild RB, Solomon H, Warty V, Kaminski DL. The effect of FK506 on canine bile flow. Transplantation 1993; 56(5); 1162-5.

39. Brems J, Reese J, Kane R, Kaminski DL. Effect of cyclosporine and steroids on canine bile flow. Hepatology 1991; 14: 523-7.

40. Kadman M, Klunemann C, Bohme M, Ishikawa T, Gorgas K, Otto G, *et al.* Inhibition by cyclosporin A of adenosine triphosphate-dependent transport from the hepatocyte into bile. Gastroenterol 1993; 104: 1507-14.

41. Sigalet DL, Kneteman NM, Thomson ABR. The effects of Cyclosporine on normal bowel. Transplantation 1991; 51(6):1296-1302.

42. Collin J, Dennison AR, Watkins RM, Millard PR, Morris PJ. Segmental small intestinal allografts: II. Inadequate function with Cyclosporine immunosuppression: Evidence of a protein-losing enteropathy. Transplantation 1987; 44(4): 479-83.

43. Sigalet DL, Kneteman NM, Simpson I, Walker K, Thomson ABR. Intestinal permeability after small intestinal transplantation and Cyclosporine treatment. Transp Proc 1992; 24(3):1120-1.

44. Sigalet DL, Kneteman NM, Fedorak RN, Kizilisik T, Madsen KE, Thomson ABR. Small intestinal function following syngeneic transplantation in the rat. 1993; Submitted.

45. Philpott DJ, Butzner JD, Meddings JB. Regulation of intestinal glucose transport. Can J Physiol Pharmacol 1992; 70: 1201-7.

46. Peters TJ, Bjarnason I. Uses and abuses of intestinhal permeability measurements. J Gastroenterol 1988; 2(3): 127-32.

47. Krentz AJ, Dousset B, Mayer D, McMaster P, Buckels J, Cramb R, *et al.* Metabolic effects of cyclosporin A and FK506 in liver transplant recipients. Diabetes 1993; 42: 1753-9.

48. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD, editors. Molecular biology of the cell. New York: Garland Publishing, Inc., 1983.

49. Williamson DH, Lund P, Krebs HA. The redox state of free nicotinamide-adenine dinucleotide in the cytoplam and mitochondria of rat liver. Biochem J 1967; 103: 514-27.

50. Henke W, Jung K. Comparison of the effects of the immunosuppressive agents FK506 and cyclosporin A on rat kidney mitochondria. Biochem Pharmacol 1993; 46(5): 829-32.

51. Mandel LJ, Bacallao R, Zampighi G. Uncoupling of the molecular 'fence' and paracellular 'gate' functions in epithelial tight junctions. Nature 1993; 361: 552-5.

CHAPTER V

RAPAMYCIN

INTRODUCTION

Rapamycin (RAPA) is a macrolide lactone which was initially isolated as an anti-fungal agent, but has been since shown to be a powerful immunosuppressive agent *in vitro* and *in vivo* (1-3). Although it is structurally related to FK506 and binds to the same intracellular protein, its mechanism of action is different (4-6). Since the late 1960's, RAPA has been found to be successful in significantly prolonging the survival of cardiac, renal and hepatic allograft survival in a number of animal models (7-14). Clinical trials with this agent are presently underway, but have not yet been published (15).

With these successes in transplantation of other vascularized organs, RAPA has come under investigation for use in small bowel transplantation (SBT). Stepkowski *et al* were among the first to demonstrate its ability to prolong survival of small bowel allograft recipients in a rat model (8, 10). Using an i.v. dose of 0.8 mg/kg/d for 14 days, mean survival time (MST) was extended to 26.8 days compared to 10.0 days in untreated controls. From semiallogeneic transplant models, it has been shown that RAPA is more effective at protecting small bowel allografts against host-versus-graft (HVG) rejection responses than against graft-versus-host-disease (GVHD) (11, 16).

More recently, significant synergism between RAPA and CsA has also been demonstrated *in vitro* (242) and *in vivo* in a rat heart allograft model (8, 9). A similar effect has been recently demonstrated with small bowel allografts, where a two week course of combined subtherapeutic doses of CsA and RAPA (0.2 mg/kg/d, i.v.) effectively prolonged the MST of untreated animals or animals treated with either agent alone, by 2 to 4-fold (12).

But, what of RAPA's adverse effects? In rats, early studies revealed that RAPA impairs weight gain, but these effects were minimal and only seen with very high doses of 50 mg/kg/d, i.m (7). More recently, however, much lower doses of 2 mg/kg/d, i.v. and 1.5 mg/kg/d, i.p. have been shown to negatively affect weight gain, from complete inhibition of growth to a 44% impairment, depending on the species of rat (18, 19). Also, although not reported in rodent models, RAPA can cause severe gastrointestinal disturbances in larger animal models of dogs and primates, even at relatively low doses. These effects include vomiting, diarrhea and the development of severe vasculitic ulcers throughout the gastrointestinal tract (7, 20, 21).

With its powerful immunosuppressive actions and promising synergism with CsA, RAPA may indeed be a potential candidate for use in SBT. However, since the success of the transplant also depends on adequate functioning of the graft to maintain nutrition and growth, these adverse effects need to be further assessed in order to define its role in SBT. The purpose of this study, therefore, is to assess growth, nutrition and small bowel function, both *in vivo* and *in vitro*, in normal rats treated with RAPA.

MATERIALS AND METHODS

ANIMALS

Juvenile male Lewis rats (275-300 g) were obtained from Charles River Canada, St. Constant, P.Q., and housed in individual plexiglass cages with free access to water and standard lab rat chow (Lab Diet, PMI Feeds, St. Louis, MO.) with an approximate composition of 23.0% protein, 4.5% fat, 6.0% fiber, 8.0% ash and 2.5% minerals. Day/night cycles were 12 hours and the temperature was maintained at $20 \pm 2°C$. Feed intake was monitored weekly, and body weight changes, biweekly. Animal care was in accordance with the guidelines of the Canadian Council of Animal Welfare (22). The experimental protocol was approved by the Animal Welfare Committee of the University of Alberta.

EXPERIMENTAL GROUPS

RAPA was suspended in carboxymethlycellulose (CMC) and administered to the test animals (n=10) at a dose of 2 mg/kg/d, injected subcutaneously (s.c.) into the nape of the neck for 6 days, followed by the same dose on alternate days for 5 more weeks. Control animals received a corresponding volume/kg of the drug vehicle, s.c., on the same schedule. As discussed in Chapter I of this thesis, the complete study undertaken was performed in two sets of experiments. This subsequently provided two control groups against which the test group could be compared: "control 1" (n=10) which was studied alongside RAPA in the first set of experiments, and an overall control group of n=20, consisting of "control 1" and "control 2", the latter (n=10) utilized during the second set of experiments. Each control group was divided in half, with 5 animals receiving CMC, and 5 receiving MCT oil, the vehicle used with the RAPA studies.

DRUGS

RAPA (a generous gift of Wyerth-Ayerst, New Haven, CT) was freshly prepared each day before injecting. An appropriate weight was first dissolved in a defined volume of 95% ethanol using a 40°C water bath, and then suspended in CMC (Sigma, St. Louis, MO.) (final vehicle composition of 9:1 CMC:ethanol) by sonication for 2 minutes, with a final concentration of 2.4 mg/ml. Drug concentrations were prepared so that equivalent volumes (0.83 ml/kg) were injected. Control animals also received equivalent volumes per kg of the liquid vehicles.

DRUG MONITORING

Whole blood RAPA levels were drawn after two weeks of drug administration by internal jugular vein puncture. Both peak (2 hours post-dose) and trough (24 hours post-dose) levels were obtained. Blood was drawn into tubes containing anticoagulant, and analyzed by high performance liquid chromatography (HPLC) as described previously (14).

IN VIVO NUTRIENT BALANCE STUDIES

After 33 days of treatment, the animals were placed in individual metabolic cages, preconditioned for 3 days, and then underwent a 3 day balance study with daily measurement of food-intake and fecal

collection. Standard lab rat chow was given *ad lib*, with a composition as described above. Total carbohydrate and fat content of feed and feces were determined using standard methods (23, 24). Briefly, feed and collected feces were completely desiccated in a freeze-dryer, and the percentage water determined directly by the change in total weight. Dried feces were then ground into a powder, and 0.2 g of sample used to determine the total energy content using a Gallenkamp Ballistic Bomb Calorimeter (London, England). O.25 g of sample was then rehydrogenated and the fat extracted into the solvent layer of a 0.9% sodium-chloride-chloroform:methanol (2:1) extraction mixture. After a second extraction of the aqueous layer, to ensure completeness, the solvent was evaporated off and the final contents weighed to determine the amount of total fat extracted. From these results, the percentages of energy and fat absorption from feed intake were calculated directly.

IN VIVO INTESTINAL PERMEABILITY

In the fifth week of the study, animals were fasted overnight and then gavaged with 100 mg of mannitol and 100 mg of lactulose in 2 ml of water during which time the animals were allowed free access to water but not food. Urine was quantitatively collected and urinary recovery of each compound was measured using high performance liquid chromatography and reported as both the percentage recovery of the total oral dose of each carbohydrate probe, and the ratio of lactulose to mannitol recovered. This latter measurement is felt to best reflect the permeability of the bowel per unit surface area (25, 26).

VILLUS MORPHOMETRY AND DENSITY

At sacrifice, sections of jejunum and ileum from control and test animals were fixed in formalin and mounted in paraffin blocks. They were cut so that both longitudinal and cross-sections of bowel were obtained which were then stained with haematoxylin and eosin. As an indirect measurement of mucosal surface characteristics, sections of ileum and jejunum, for each group, were photomicrographed at a magnification of 100X. Morphometric analyses were performed using an Image analysis system (Joyce Loebl, Magiscan) (27). Image analysis systems have been used to efficiently determine morphometric parameters of large numbers of intestinal villi in several recent studies analyzing intestinal mucosal

architecture (28, 29). Parameters studied included villus length, as measured from the lamina propria, width and overall sagittal section area for 25 villi per histological section. Three sections from both the jejunum and ileum for each test group and four sections from both the jejunum and ileum of the controls were analyzed, resulting in 75 to 100 villi being measured per group for each region of small bowel. The number of villi per microscopic field (at 20X magnification) were determined as a measure of villus density.

IN VITRO GLUCOSE FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

After 6 weeks of treatment, animals were sacrificed with an intraperitoneal pentobarbital overdose and *in vitro* small bowel function studies were performed. Segments of distal ileum and proximal jejunum, taken approximately 2 to 3 cm from the ileocecal valve and ligament of Trietz, respectively, were quickly excised, rinsed with ice-cold normal Ringers solution, and stored in ice-cold Ringers gassed with carbogen (5% CO_2 in O_2) during the preparation for mounting onto Ussing chambers as previously described (30, 31). Briefly, the intestine was split along the mesenteric border and segments of approximately 2 cm were then quickly stripped of the serosa and underlying muscle layers and then clamped into Ussing chambers . This subsequently divides the chamber into mucosal and serosal compartments. Tissues were bathed on both sides by normal Ringers (Na^+, 143 mM; K^+, 5 mM; Ca^{2+}, 1.25 mM; Mg^{2+} 1.1 mM; Cl^-, 123.7 mM; HCO_3, 25 mM; $HPO_4 + H_2PO_4$, 1.95 mM) with 20 mM fructose, and gassed with carbogen to pH of 7.4. A circulating water bath maintained the chamber temperature at 37°C. For measurement of unidirectional fluxes, 3-O-methyl-D-glucose (3OMeG) was present at a concentration of 20 mM on both mucosal and serosal sides and 5 μCi of [^{3}H]-3OMeG was added to either the mucosal or serosal side of the tissue. To ensure steady-state conditions, tissues were maintained under these conditions for 20 minutes. Previous studies have shown that glucose flux is constant over this time period (31). Unidirectional mucosal-to-serosal (J_{ms}) and serosal-to-mucosal (J_{sm}) fluxes were measured in paired tissues by collection of aliquots of incubation solution from each side of the chambers and determining the radioactivity in a Beckman LS5801 β-Scintillation Counter (California). Tissue pairs

were discarded if electrical conductance (see below) varied by >15%. Four consecutive 5 minute fluxes were determined. Tissue response to 5 mM theophylline was used to confirm viability at the completion of the experiment.

Electrophysiological parameters were monitored throughout. The spontaneous transepithelial electrical potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (I_{sc}) with an automatic voltage clamp (DCV 1000 World Precision Instruments, New Haven, CT), except for 3-5 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue conductance (G) was calculated from potential difference and I_{sc} according to Ohm's law, where voltage (V) = current (I_{sc}) x resistance (R) and conductance (G) is the reciprocal of R (30).

STATISTICAL ANALYSES

Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using a computerized statistical package (SigmaStat Statistical Analysis System, Jandel Corporation). Means between the two groups (controls vs treatment) were compared using the Student's t-test unless the distribution was nonparametric (as determined directly by the program, using the Kolmogorov-Smirnov test of normality, with a p value of >0.05) in which case a distribution-free test comparing the medians is more appropriate, and was performed with a Mann-Whitney rank sum test. A p-value of <0.05 was considered statistically significant. No statistically significant differences were determined between the MCT and CMC treatments, and in all but three sets of results (stool water content, lactulose/mannitol permeability and villus morphometry), no differences were found between the two control groups ("control 1" and "control 2"). Subsequently, all control animals were combined into one control group with n=20. In studies where the two significantly differed , the differences were assumed to be secondary to altered laboratory processing of the samples, and the RAPA group was compared to its respective "control 1" group, only.

RESULTS

WEIGHT GAIN, FEED INTAKE & *IN VIVO* NUTRITIONAL BALANCE STUDIES

Rate of weight gain over the six week period was significantly impaired in the treated animals, with a 73% reduction relative to control animals. Feed intake was significantly reduced, but only by 6% relative to controls. These effects are tabulated in Table V-1.

OTHER GASTROINTESTINAL EFFECTS

The treated animals developed moderately inspissated stools, with a significant 33% decrease in stool water content compared to controls ($p=0.005$).

DRUG LEVELS

Mean peak and trough levels of RAPA obtained after two weeks of drug administration were 52 ± 9 ng/ml and 24 ± 2 ng/ml, respectively.

VILLUS MORPHOMETRY AND DENSITY

Except for a small, albeit significant, 12% increase in villus height, jejunal villus morphometry and density showed little change relative to controls. In the ileum, however, the villi appeared markedly blunted, with mean height and width significantly reduced by 18% and 16%, respectively. This resulted in a 36% decrease in mean saggital section area. Although the villus density did increase by 19% in this region of bowel, this did not reach statistical significance ($p=0.187$). These results are shown in Table V-3 and displayed in Plate 2 (Appendix I).

IN VIVO BOWEL PERMEABILITY

RAPA-treated animals demonstrated significant decreases in both mannitol and lactulose recoveries, by 49% and 30%, respectively. The lactulose/mannitol ratio, however, was not significantly altered. (Table V-2).

IN VITRO 3OMeG FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

The effects of RAPA on *in vitro* 3OMeG transmural fluxes are summarized in Table V-4. No significant differences compared to controls were noted in the jejunum except for a 65% increase in I_{sc}. In

the ileum, the unidirectional J_{ms} was decreased while the J_{sm} was increased, both insignificantly, however. Nonetheless, this resulted in a significant obliteration of J_{net} to zero.

DISCUSSION

The marked weight loss occurring in the treated animals indicates significant toxicity of RAPA at the dose used. Similar weigh loss effects have been described by others. A dose of 2 mg/kg/d, i.v. severely affects weight gain, from complete inhibition of growth to a 73% impairment (18). A lower dose of 1.5 mg/kg/d, i.p. had a lesser effect, but still decreased weight gain by 44%, relative to control animals (19). Unfortunately, the optimal dose of RAPA in the rat model of SBT has not yet been determined. Stepkowski *et al* were able to achieve significant prolongation of allografts with doses of 0.8 mg/kg/d, i.v for 2 weeks, but once the agent was discontinued, rejection ensued (8,10,12). Lower doses of 1 mg/kg on alternate days, i.m., for 1 week, were ineffective (32). More recently, as developed from experience with CsA and FK506, it has been determined that maintenance of appropriate blood concentrations of the drug are more important for therapeutic efficacy in order to optimize RAPA's use . In canine islet and rabbit cardiac transplantation models, it has been determined that maintenance of trough concentration between 10-60 μg/L allows for optimal prolongation of graft survival while minimizing toxicity (21). Although these optimal levels may be species-specific, similar levels were obtained in our study

This profound effect on weight gain could not be completely attributed to the small, albeit significant, 6% decrease in feed intake in the treated animals. Nor is it likely to be the result of a 5% reduction in fat absorption while energy absorption was unchanged. Except for a small increase in villus height, villus size and density were comparable to controls in the jejunum of treated animals. As the jejunum is the major site of nutrient absorption in the small intestine, the impaired weight gain is also unlikely to be the result of changes in mucosal surface area of the ileum.

The marked villus blunting observed in the ileum has not been previously described. Interestingly, however, Francavilla *et al* have shown that RAPA significantly inhibits small intestinal regeneration after

a 40% resection, excluding the jejunum (33). Our studies indicated a ileum-specific effect, however, further studies, perhaps looking at RAPA's effects on regeneration of segmental resections from varying regions of the bowel, may clarify this. As the ileum is not the primary site of nutrient absorption, these changes are unlikely to be the primary cause of the severe effect seen on weight gain. However, a negative effect on enterocyte function in the ileum is suggested by the significant obliteration of net glucose uptake in this region of bowel. The ileum is responsible for bile salt uptake as part of the enterohepatic circulation, and enterocyte dysfunction in the region may thereby reduce the bile acid pool and contribute to the fat malabsorption observed (34). Although anemia was not obvious in these studies, future studies should investigate vitamin B12 absorption, a function also specific to the ileum.

Changes in intestinal permeability secondary to RAPA are difficult to assess in these studies. Both mannitol and lactulose recoveries were significantly decreased. However, with no change in lactulose/mannitol ratio, relative to controls, this suggests that these results are more likely secondary to rapid transit of the probes through the small intestine rather than direct changes in permeability. The lack of significant changes in electrical conductance in both the jejunum and ileum supports this conclusion.

Indeed, except for the changes already noted in the ileum, the poor weight gain in the RAPA-treated animals was not fully attributable to any of the small bowel functions we studied. In the jejunum, we assessed enterocyte function by *in vivo* 3OMeG uptake studies. Neither mucosal-to-serosal flux (J_{ms}), nor net flux (J_{net}) were significantly altered. There was a significant 65% increase in mean I_{sc} recorded. However, this did not correspond to any of the flux data or other electrophysiological parameters, and is therefore unexplainable at this time.

The adverse effect of RAPA on weight gain in these animals, rather than being due to a change in intestinal function, may be related to the proposed mechanism of action of this agent. Upon combining with its known intracellular binding-protein, the FK506-binding-protein (FKBP). This rapamycin-FKBP complex strongly inhibits the activation of the 70 kd S6 protein kinase (p70 S6), which is presumably involved in regulation of protein synthesis (35-37,38). Although most studies have investigated this

action with respect to the role of p70 S6 in the signaling pathway of T-cell activation (37). RAPA has also been found to be able to inhibit cell proliferation of other cell lines, such as mast cells (38), fibroblasts (35) and myocytes (39). Therefore, it could potentially have more global effects with respect to protein synthesis required for the maintenance of overall body cell mass.

In summary, RAPA severely impairs weight gain. This cannot be entirely explained by decreases in feed intake, nor a small decrease in fat absorption. At the small bowel level, although significant villus blunting occurs in the ileum, no adverse effects are noted in the jejunum. Functionally, net glucose uptake appears to be obliterated in the ileum while this function of the enterocyte was unchanged in the jejunum. Finally, the relationship of RAPA's proposed mechanism of action--inhibition of p70 S6 protein kinase--to its effects on growth, structural and intestinal function needs to be established before considering it for transplantation of the small intestine.

Table V-1

Effects of Rapamycin on Weight gain, Feed intake and Energy and Fat Absorption

	Weight Gain (g/d)	Feed Intake (g/kg/d)	% Energy Absorption	% Fat Absorption
Control (n=20)	3.15 ± 0.07	69.42 ± 1.11	81.3 ± 0.6	76.1 ± 0.9
Rapamycin (n=10)	0.84 ± 0.08 ***73%↓** $p < 0.001$	65.16 ± 0.66 ***6%↓** $p = 0.002$	80.1 ± 1.5 1%↓ $p = 0.345$	71.9 ± 2.2 ***5%↓** $p = 0.045$

Values are means ± SEM.

***** significant difference compared to controls ($p<0.05$); compared with student's t-test.

% values indicated relative to controls (↑, increase; ↓, decrease).

Table V-2

Effects of Rapamycin on *In Vivo* Intestinal Permeability

	Mannitol	Lactulose	Lactulose/ Mannitol
Control 1 (n=10)	3.77 ± 0.22	1.40 ± 0.11	0.41 ± 0.03
Rapamycin (n=10)	1.94 ± 0.26 ***49%↓** p<0.001	0.98 ± 0.12 ***30%↓** p 0.027	0.48 ± 0.04 16%↑ p 0.083

Data is presented as percent recovery of orally administered marker in urine; mean ± SEM.
* significant difference compared to controls ($p<0.05$); compared with student's t-test.
% values indicated relative to controls (↑, increase; ↓, decrease).

Table V-3
Effects of Rapamycin on Villus Morphometry and Density

	Width ($\mu m \times 10^2$)	Height ($\mu m \times 10^2$)	Area ($\mu m^2 \times 10^2$)	# Villi per 20X field
JEJUNUM				
Control 1 (n=10)	1.66 ± 0.05	4.74 ± 0.12	4.67 ± 0.19	6.58 ± 0.52
Rapamycin (n=10)	1.55 ± 0.04 6%↓ p=0.212	5.32 ± 0.10 ***12%↑** p<0.001	4.97 ± 0.16 6%↑ p=0.289	6.65 ± 0.42 1%↑ p=0.928
ILEUM				
Control 1 (n=10)	1.70 ± 0.06	3.84 ± 0.14	4.13 ± 0.23	5.26 ± 0.41
Rapamycin (n=10)	1.42 ± 0.07 ***16%↓** p<0.001	3.14 ± 0.04 ***18%↓** p=0.007	2.65 ± 0.12 ***36%↓** p<0.001	6.27 ± 0.54 19%↑ p=0.187

Data is presented as villus width, height from lamina propria and saggital section area; expressed as mean ± SEM.
* significant difference compared to controls ($p<0.05$); compared with student's t-test.
% values indicated relative to controls (↑, increase; ↓, decrease).

Table V-4
Effects of Rapamycin on *In Vitro* 3OMeG fluxes and Electrophysiological Parameters

	J_{ms}	J_{sm}	J_{net}	PD	I_{sc}	G
	JEJUNUM					
Control (n=20)	1.24 ± 0.08	0.84 ± 0.06	0.41 ± 0.05	1.70 ± 0.12	39.29 ± 3.16	22.56 ± 1.54
Rapamycin (n=10)	1.35 ± 0.06 9%↑ p=0.632	0.78 ± 0.17 7%↓ p=0.742	0.57 ± 0.21 39%↑ p=0.291	1.71 ± 0.08 1%↑ p=0.998	64.78 ± 6.63 ***65%↑** p=0.002	5.98 ± 5.56 15%↑ p=0.471
	ILEUM					
Control (n=20)	1.19 ± 0.08	0.82 ± 0.08	0.36 ± 0.06	2.26 ± 0.15	40.86 ± 2.59	18.25 ± 1.50
Rapamycin (n=10)	1.06 ± 0.13 11%↓ p=0.436	1.07 ± 0.14 30%↑ p=0.125	-0.01 ± 0.07 ***100%↓** p<0.001	2.12 ± 0.34 6%↓ p=0.482	37.56 ± 2.16 8%↓ p=0.378	20.82 ± 1.22 14%↑ p=0.228

Values are means ± SEM. J_{ms}, flux from mucosa to serosa; J_{sm}, flux from serosa to mucosa; J_{net}, numerical difference between J_{ms} and J_{sm} in μmol/cm^2/hr; PD, potential difference in mV; Isc, short-circuit current in μAmp/cm^2/hr; G, conductance in mS/cm^2.

*significant difference compared to controls ($p<0.05$); compared with student's t-test; % values indicated relative to controls (↑, increase; ↓, decrease).

REFERENCES

1. Sehgal SN, Baker H, Vezina C. Rapamycin (A4-22.989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. J. Antibiot 1975; 28: 727-32.

2. Martel RR, Klicius J, Galet S. Inhibition of the immune response by rapamycin, a new antifungal antibiotic. Can J. Physiol Pharmacol 1977; 55: 48-51.

3. Morris RE. Rapamycins: Antifungal, antitumour, antiproliferative and immunosuppressive macrolides. Transplant Rev 1992; 6: 39.

4. Dumont FJ, Melino MR, Staruch MJ, Koprak SL, Fischer PA, Sigal NH. The immunosuppressive macrolides FK506 and rapamycin act as reciprocal antagonists in murine T-cells. J. Immunol 1990; 144: 1418-24.

5. Bierer BE, Marrila PS, Standaert RF, Herzenberg LA, Burakoff SJ, Crabtree, Schreiber SL. Two distinct signal tranmission pathways in T-lymphocyes are inhibited by complexes formed between an immunophilin and either FK506 or Rapamycin. Proc Natl Acad Sci, USA 1990; 87: 9231-5.

6. Kunz J, Hall MN. Cyclosporin A, FK506 and rapamycin: More than just immunosuppression. TIBS 1993; 18(9): 334-8.

7. Calne RY, Lim S, Samaan A, St.J. Collier D, Pollard SG, White DJG, Thiru S. Rapamycin for immunosuppression in organ allografting. Lancet 1989; 2: 227.

8. Stepkowski SM, Chen H, Daloze P, Kahan BD. Prolongation by Rapamycin of heart, kidney, pancreas, and small bowel allograft survival in rats (+ synergistic effect with Cyclosporine). Transplant Proc 1991; 23(1): 507-8.

9. Morris RE, Meiser BM, Wu J, Shorthouse R, Wang J. Use of Rapamycin for the suppression of alloimmune reactions in vivo: Schedule dependence, tolerance induction, synergy with cyclosporine and FK506, and drrect on host-versus-graft and graft-versus-host reactions. Transplant Proc 1991; 23(1): 521-4.

10. Stepkowski SM, Chen H, Daloze P, Kahan BD. Rapamycin, a potent immunosuppressive drug for vascularized heart, kidney, and amall bowel transplantation in the rat. Transplantation 1991; 51(1): 22-26.

11. Stepkowski SM, Chen HF, Wang ME, Daloze P, Kahan BD. Inhibition of host-versus-graft and graft-versus-host responses after small bowel transplantation in rats by Rapamycin. Transplantation 1992; 53(2): 258-64.

12. Chen H, Wu J, Xu D, Aboujaoude M, Stepkowski S, Kahan B, Daloze P. The effect of rapamycin on orthotopic small bowel transplantation in the rat. Transplant Proc 1992; 24(3): 1157-8.

13. Chen H, Wu J, Xu D, Luo H, Daloze PM. Reversal of ongoing heart, kidney, and pancreas allograft rejection and suppression of accelerated heart allograft rejection in the rat by rapamycin. Transplantation 1993; 56(3): 661-6.

14. Fryer J, Yatscoff RW, Pascoe EA, Thliveris J. Relationship of blood concentrations of rapamycin and cyclosporine to suppression of allografts rejection in a rabbit heterotopic heart transplant model. Transplantation 1993; 55(2): 340-5.

15. Yatscoff RW, LeGatt DF, Kneteman NM. Therapeutic monitoring of rapamycin: A new immunosuppressive drug. Ther Drug Monit 1993; 15(6): 478-82.

16. Fabian MA, Denning SM, Bollinger RR. Rapamycin suppression of host-versus-graft and graft-versus-host disease in MHC-mismatched rats. Transplant Proc 1992; 24(3): 1174.

17. Kahan BD, Gibbons S, Tejpal N, Stepkowski SM, Chou T-C. Synergistic interactions of Cyclosporine and Rapamycin to inhibit immune performances of normal human peripheral blood lymphocytes in vitro. Transplantation 1991; 51: 232-8.

18. DiJoseph J, Mihatsch M, Sehgal S. Influence of rat strain on rapamycin's kidney effects. Transplant Proc 1993; 25(1): 6714-5.

19. Whiting PH, Woo J, Adam BJ, Hasan NU, Davidson RJL, Thomson AW. Toxicity of Rapamycin - A comparative and combination study with Cyclosporin at immunotherapeutic dosage in the rat. Transplantation 1991; 52(2): 203-8.

20. St. J. Collier D, Calne RY, Pollard SG, Friend PJ, Thiru S. Rapamycin in experimental renal allografts in primates. Transplant Proc 1991; 23(4): 2246-7.

21. Yakimets WJ, Katyal D, Lakey JR, Yatscoff RW, Ao Z, Rajotte RV, Kneteman NM. Combination low dose rapamycin and cyclosporine prolong canine pancreatic islet graft survival. Transplantation (in press).

22. Canadian Council on Animal Care. Guide to the care and use of Experimental Animals. The Council, 1980.

23. Association of Official Analytical Chemist (1986): Methods of analysis (23 Ed.). AOAC, Washington, D.C.

24. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1956; 226: 497-509.

25. Elia M, Behrens R, Northrop C, Wraight P, Neale G. Evalation of mannitol, lactulose and ^{51}Cr-labelled ethylenediaminetetra-acetate as markers of intestinal permeability in man. Clin Sci 1987; 73: 197-204.

26. Hamilton I, Rothwell J, Archer D, Axon ATR. Permeability of the rat small intestine to carbohydrate probe molecules. Clin Sci 1987; 73: 189-96.

27. Joyce Loebl. Image analysis: Principles and practice. Great Britain: Joyce Loebl, 1985.

28. Erickson RA, Rivera N. Effect of difluoromethylornithine (DFMO) on NSAID-induced intestinal injury in rats. Dig Dis Sci 1992; 37(12): 1833-9.

29. Pahl MV, Erickson RA, Vaziri ND, Khamiseh G, Oveisi F, Jan K, *et al.* Intestinal morphometry and bile acid-induced mucosal injury in chronic experimental renal failure. J Lab Clin Med 1990; 115: 572-8.

30. Field M, Fromm D, McColl I. Ion transport in rabbit ileal mucosa. I. Na and Cl fluxes and short-circuit current. Am J Physiol 1971; 220(5): 1388-96.

31. Fedorak RN, Chang EB, Madara JL, Field M. Intestinal adaptation to diabetes: Altered Na-dependent nutrient absorption in streptozocin-treated chronically diabetic rats. J Clin Invest 1987; 79: 1571-8.

32. Marquet RL, de Bruin R, Heineman E, Jeekel J. Efficacy of Rapamycin in orthotopic small bowel transplantation in the rat. Transplant Proc 1993; 25(4): 2695-6.

33. Francavilla A, Starzl TE, Scotti C, Carrieri G, Azzarone A, Zeng QH, *et al.* Inhibition of liver, likney, and intestine regeneration by Rapamycin. Transplantation 1992; 53(2): 296-8.

34. Thomson ABR, Shaffer EA, editors. First principles of gastroenterology: The basis of disease and an approach to management. Canadian Association of Gastroenterology: Toronto, 1992.

35. Shan X, Luo H, Chen H, Daloze P, St-Louis G, Wu J. The effect of rapamycin on c-jun expression in human lymphocytes. Clin Immunol Immunopath 1993; 69(3): 314-7.

36. Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the p70 kd S6 protein kinases. Cell 1992; 69: 1227-36.

37. Kuo CJ, Chung J, Fiorentinao DF, Flanagan WM, Blenis J, Crabtree GR. Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. Nature 1992; 358: 70-3.

38. Tsai M, Chen R-H, Tam S-Y, Blenis J, Galli SJ. Activation of MAP kinases, $pp90^{rsk}$ and pp70-S6 kinases in mouse mast cells by signaling through the c-*kit* receptor tyrosine kinase or $Fc_{\varepsilon}RI$: rapamycin inhibits activation of pp70-S^ kinase and proliferation in mouse mast cells. Eur J immunol 1993; 23: 3286-91.

39. Jayaraman T, Marks AR. Rapamycin-FKBP12 blocks proliferation, induces differentiation, and inhibits cdc2 kinase activity in a myogenic cell line. J Biol Chem 1993; 268(34): 25385-8.

CHAPTER VI

DEOXYSPERGUALIN

15-Deoxyspergualin (DSG) is a potent immunosuppressive agent, originally developed as an antitumor agent (1). Not only is it structurally different from cyclosporin A (CsA), FK506 and rapamycin, but it appears to effect its immunosuppressive actions later on in the rejection cascade, primarily by suppressing proliferation and differentiation of activated T-lymphocytes as well as impair humoral immunity (2-6). In animal models, DSG has been shown to prolong cardiac, renal hepatic, pancreatic and thyroid allografts with a wide range of doses (7-15). More recently, DSG has been utilized in the treatment of acute rejection in clinical renal allografts (16-18) and has proven successful as induction therapy for pancreatic islet transplantation (19). DSG appears to be most effective, however, when used in combination with CsA, where the synergistic effect of the two agents is significantly more effective in prolonging rat cardiac and pancreaticoduodenal allograft survival than either agent alone (10,20,21).

To date, limited studies have been published investigating the efficacy of DSG on SBT. However, Tanaka *et al* were able to demonstrate its ability to prevent the development of cutaneous or lethal GVHD in a one-way GVHD rat model when administered for 10 days post SBT (5 mg/kg/d, i.p.) (22). More recently, in a swine SBT model, 3 mg/kg/d, i.v., for 1 week followed by therapy at half this dose, allowed for significant prolongation of allograft survival, although it was not as efficacious as immunosuppression with CsA (23).

In rats, side effects of DSG include weight loss, bone marrow toxicity and infectious complications (4). Doses of 5 mg/kg/d, i.m. were shown by Jindal *et al* to be toxic to rats after 1-2 weeks of treatment, with marked weight loss, diarrhea, patchy hair loss, lethargy, nosebleeds, sepsis and evidence of

respiratory distress (24). Mice treated with 2.5 to 5 mg/kg/d, i.p. for 2 or more weeks also develop significant but reversible weight loss as well as anemia, leukopenia and alterations of hematopoietic and lymphoid tissues (9, 11, 25). Larger animals such as dogs and swine appear to be more sensitive to DSG with the development of severe gastrointestinal disturbances such as diarrhea, bleeding, and emaciation, even in a minimally immunosuppressive dose of 0.6 to 1.8 mg/kg/d. (13, 15, 26).

In humans, toxic effects appeared to be dose-dependent, including facial numbness, headache, infectious complications, and most significantly, alterations of the hematopoietic system with leukopenia, thrombocytopenia, and erythrocytopenia, occurring with doses of 3-5 mg/kg/d over 5-10 days (17). Subjective complaints of gastrointestinal disturbances, including abdominal distention, upper abdominal discomfort, epigastric pain and heartburn, are also described (18). None of these adverse effects, however, are usually severe enough to warrant discontinuation of the drug (17).

To date, there have been no published studies of DSG being used in clinical SBT. However, its successes in animal models of other vascularized organs and in clinical renal and islet transplantation and its alternate mechanism of action compared to more conventional immunosuppressive agents and its versatility in organ transplantation, makes it an attractive alternative to be investigated for use in SBT. However, the observed toxicities of weight loss, gastrointestinal disturbances and diarrhea suggest caution. We felt, therefore, that it is important to look more closely at these effects, specifically assessing nutritional indices and *in vivo* and *in vitro* small bowel function.

MATERIALS AND METHODS

ANIMALS

Juvenile male Lewis rats (275-300 g) were obtained from Charles River Canada, St. Constant, P.Q., and housed in individual plexiglass cages with free access to water and standard lab rat chow (Lab Diet, PMI Feeds, St. Louis, MO.) with an approximate composition of 23.0% protein, 4.5% fat, 6.0% fiber, 8.0% ash and 2.5% minerals. Day/night cycles were 12 hours and the temperature was maintained at

20 ± 2°C. Feed intake was monitored weekly, and body weight changes, biweekly. Animal care was in accordance with the guidelines of the Canadian Council of Animal Welfare (27). The experimental protocol was approved by the Animal Welfare Committee of the University of Alberta.

EXPERIMENTAL GROUPS

The test animals (n=10) received subcutaneous (s.c.) injections into the nape of the neck of DGS suspended in carboxymethylcellulose (CMC). 2 mg/kg qd for 6 days, followed by 2 mg/kg on alternate days for 5 more weeks. Control animals received a corresponding volume/kg of the drug vehicle, on the same schedule. As discussed in the "Introduction" of this thesis, the complete study undertaken was performed in two sets of experiments. This subsequently provided two control groups against which the test group could be compared including "control 2" (n=10) which was studied alongside DSG in the second set of experiments, and an overall control group of n=20 consisting of "control 2" and "control 1" (n=10), which was utilized in the first set of experiments. Each control group was divided in half, with 5 animals CMC, and 5 receiving MCT oil, the vehicle used with the CsA studies.

DRUGS

DSG, a generous gift of Bistol-Meyers Squibb Pharmaceutical Research Institute, CT, was dissolved in one part normal saline and then further diluted with 9 parts CMC (Sigma, St. Louis, MO.), to a final concentration of 3.6 mg/ml. It was freshly prepared every week and stored in the dark at 4°C. Drug concentrations were prepared so that equivalent volumes (0.83 ml/kg) were injected throughout the study. Control animal received and equivalent volume per kg of the CMC vehicle.

HEMATOLOGICAL PARAMETERS

Complete blood counts were determined on each animal at sacrifice using a M430 Coulter Counter. Electrolytes, creatinine and blood glucose levels were also determined using a Beckman Astra-Eight analyzer.

HYPERREACTIVITY STUDIES

Subjective observations of increased aggression in the test rats was quantitated and compared with controls using a modified version of a reactivity test for rats as described by Albert and Richmond (28). Briefly, the animals' response to 6 test stimuli were rated on a scale of 0 to 3, with 0 indicating no response and 3 indicating attack or highly aggressive response (vocalization and/or biting). Test items were: 1) Presentation of a pencil just in front of the rat's snout; 2) A sharp tap on the back with a pencil; 3) Presentation of a gloved hand before the snout; 4) Gentle prods on the side of the rat's body with a blunt one inch diameter stick; 5) Attempted capture by the tail; and 6) Attempted grasping around the body. The total score was recorded, with a maximum reactivity score possible of 18.

IN VIVO NUTRIENT BALANCE STUDIES

After 33 days of treatment, the animals were placed in individual metabolic cages, preconditioned for 3 days, and then underwent a 3 day balance study with daily measurement of food-intake and fecal collection. Standard lab rat chow was given *ad lib*, with a composition as described above. Total carbohydrate and fat content of feed and feces were determined using standard methods (29,30). Briefly, feed and collected feces were completely desiccated in a freeze-dryer, and the percentage water determined directly by the change in total weight. Dried feces were then ground into a powder, and 0.2 g of sample used to determine the total energy content using a Gallenkamp Ballistic Bomb Calorimeter (London, England). 0.25 g of sample was then rehydrogenated and the fat extracted into the solvent layer of a 0.9% sodium-chloride-chloroform:methanol (2:1) extraction mixture. After a second extraction of the aqueous layer, to ensure completeness, the solvent was evaporated off and the final contents weighed to determine the amount of total fat extracted. From these results, the percentages of energy and fat absorption from feed intake were calculated directly.

IN VIVO INTESTINAL PERMEABILITY

One day following the balance studies, animals were fasted for 6 hours and then gavaged with 200 μCi of ^{99}Tc-DTPA in 1.5 ml of water. Urine was quantitatively collected over 20 hours while the animals were allowed *ad lib* access to food and water. The percent urinary recovery of the labeled probe was determined by simultaneously counting aliquots of the original test solution and urines in a γ-counter, both having undergone the same period of radioactive decay.

On the following day, animals were fasted overnight and then gavaged with 100 mg of mannitol and 100 mg of lactulose in 2 ml of water during which time the animals were allowed free access to water but not food. Urine was quantitatively collected and urinary recovery of each compound was measured using high performance liquid chromatography and reported as both the percentage recovery of the total oral dose of each carbohydrate probe, and the ratio of lactulose to mannitol recovered. This latter measurement is felt to best reflect the permeability of the bowel per unit surface area (31,32).

VILLUS MORPHOMETRY AND DENSITY

At sacrifice, sections of jejunum and ileum from control and test animals were fixed in formalin and mounted in paraffin blocks. They were cut so that both longitudinal and cross-sections of bowel were obtained which were then stained with haematoxylin and eosin. As an indirect measurement of mucosal surface characteristics, sections of ileum and jejunum, for each group, were photomicrographed at a magnification of 100X. Morphometric analyses were performed using an Image analysis system (Joyce Loebl, Magiscan) (33). Image analysis systems have been used to efficiently determine morphometric parameters of large numbers of intestinal villi in several recent studies analyzing intestinal mucosal architecture (34,35). Parameters studied included villus length, as measured from the lamina propria, width and overall sagittal section area for 25 villi per histological section. Three sections from both the jejunum and ileum for each test group and four sections from both the jejunum and ileum of the controls were analyzed, resulting in 75 to 100 villi being measured per group for each region of small bowel. The

number of villi per microscopic field (at 20X magnification) were determined as a measure of villus density.

IN VITRO GLUCOSE FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

After 6 weeks of treatment, animals were sacrificed with an intraperitoneal pentobarbital overdose and *in vitro* small bowel function studies were performed. Segments of distal ileum and proximal jejunum, taken approximately 2 to 3 cm from the ileocecal valve and ligament of Trietz, respectively, were quickly excised, rinsed with ice-cold normal Ringers solution, and stored in ice-cold Ringers gassed with carbogen (5% CO_2 in O_2) during the preparation for mounting onto Ussing chambers as previously described (36,37). Briefly, the intestine was split along the mesenteric border and segments of approximately 2 cm were then quickly stripped of the serosa and underlying muscle layers and then clamped into Ussing chambers . This subsequently divides the chamber into mucosal and serosal compartments. Tissues were bathed on both sides by normal Ringers (Na^+, 143 mM; K^+, 5 mM; Ca^{2+}, 1.25 mM; Mg^{2+} 1.1 mM; Cl^-, 123.7 mM; HCO_3, 25 mM; $HPO_4 + H_2PO_4$, 1.95 mM) with 20 mM fructose, and gassed with carbogen to pH of 7.4. A circulating water bath maintained the chamber temperature at 37°C. For measurement of unidirectional fluxes, 3-O-methyl-D-glucose (3OMeG) was present at a concentration of 20 mM on both mucosal and serosal sides and 5 μCi of [^{3}H]-3OMeG was added to either the mucosal or serosal side of the tissue. To ensure steady-state conditions, tissues were maintained under these conditions for 20 minutes. Previous studies have shown that glucose flux is constant over this time period (37). Unidirectional mucosal-to-serosal (J_{ms}) and serosal-to-mucosal (J_{sm}) fluxes were measured in paired tissues by collection of aliquots of incubation solution from each side of the chambers and determining the radioactivity in a Beckman LS5801 β-Scintillation Counter (California). Tissue pairs were discarded if electrical conductance (see below) varied by >15%. Four consecutive 5 minute fluxes were determined. Tissue response to 5 mM theophylline was used to confirm viability at the completion of the experiment.

Electrophysiological parameters were monitored throughout. The spontaneous transepithelial electrical potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (I_{sc}) with an automatic voltage clamp (DCV 1000 World Precision Instruments, New Haven, CT), except for 3-5 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue conductance (G) was calculated from potential difference and I_{sc} according to Ohm's law, where voltage (V) = current (I_{sc}) x resistance (R) and conductance (G) is the reciprocal of R (37).

ENTEROCYTE METABOLIC FUNCTION

At sacrifice, enterocytes were isolated from the distal jejunum, tested for viability and analyzed with respect to mitochondrial substrate utilization and energy production as described elsewhere (38). Briefly, after isolation of the cells, aliquots were incubated for 60 minutes in the presence of either [^{14}C]glucose or 2-[^{14}C]pyruvate and the amount of each substrate utilized quantitated by the amount of [^{14}]CO_2 released (39). Lactate and pyruvate production was then determined from aliquots of cells over a 60 minute incubation period and reported as mg/h/mg protein. Finally, intracellular ATP levels were quantitated after extraction from cell suspensions, using the luciferase/Luciferin assay kit (Sigma Diagnostics) in a Lumat lumnometer.

STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using a computerized statistical package (SigmaStat Statistical Analysis System, Jandel Corporation). Means between the two groups (controls vs treatment) were compared using the Student's t-test unless the distribution was nonparametric (as determined directly by the program, using the Kolmogorov-Smirnov test of normality, with a p value of >0.05) in which case a distribution-free test comparing the medians is more appropriate, and was performed with a Mann-Whitney rank sum test. A p-value of <0.05 was considered statistically significant. No statistically significant differences were determined between the

MCT and CMC treatments, and in all but three sets of results (stool water content, lactulose/mannitol permeability and villus morphometry), no differences were found between the two control groups ("control 1" and "control 2"). Subsequently, all control animals were combined into one control group with n=20. In studies where the two significantly differed , the differences were assumed to be secondary to altered laboratory processing of the samples, and the DSG group was compared to its respective "control 2" group, only.

RESULTS

WEIGHT GAIN, FEED INTAKE & *IN VIVO* NUTRITIONAL BALANCE STUDIES

The rate of weight gain over the six week period was significantly reduced, by 26%, in those animals receiving DSG. Feed intake, however, was comparable to controls. These effects are tabulated in Table VI-1.

IN VIVO NUTRITIONAL BALANCE STUDIES

Fat absorption was significantly decreased by 12% ($p<0.021$) relative to controls. Energy absorption (kcal) was not altered (Table VI-1).

OTHER EFFECTS

The treated animals developed mild but significant diarrhea, as manifested by a 34% increase in stool water content compared to controls ($p<0.001$). Alopecia, most marked over the facial region, was noted after two to three weeks of therapy, and progressed until the end of the study.

SERUM & HEMATOLOGICAL PARAMETERS

Serum creatinine was significantly increased by 45% although K+ levels were comparable to controls. Likewise, random serum glucose levels were not altered. The animals developed anemia and leukopenia, with RBC, hemoglobin and hematocrit significantly reduced by 25-32% compared to controls, and WBC by 43% (Table VI-2).

HYPERREACTIVITY TESTING

Moderately aggressive behavior and hyperreactivity was observed in the treated animals after 2 to 3 weeks of drug administration, and persisted until sacrifice. Testing at 5 weeks revealed a significantly increased hyperreactivity score (mean score of 4.8 ± 0.5) compared to controls (mean score of 3.0 ± 0.3, $p<0.05$).

IN VIVO BOWEL PERMEABILITY

Percent recovery of ^{99}Tc-DTPA was increased by 66% in the DSG animals, this value almost reaching statistical significance ($p=0.55$). Permeability to both lactulose and mannitol, however, were comparable to controls, as was the lactulose/mannitol ratio (Table VI-3).

VILLUS MORPHOMETRY AND DENSITY

A 13-14% decrease in mean saggital section area of the villi in both jejunal and ileal sections was noted in the DSG group, indicating a small but significant reduction in overall villus size. There were no significant changes, however, in villus density (Table VI-4).

IN VITRO 3OMeG FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

Electrical conductance was significantly increased by 33% in the ileum as shown in Table VI-5. This was associated with a 6% increase in serosal-to-mucosal 3OMeG flux (J_{sm}) and 12% increase in mucosal-to-serosal flux (J_{ms}). However, none of the changes in 3OMeG fluxes reached statistical significance.

ENTEROCYTE METABOLIC FUNCTION

Enterocytes isolated from animals treated with DSG produced 61% less CO_2 from glucose when incubated in the presence of 20 mM D-glucose as compared with enterocytes isolated from controls. The release of $^{14}CO_2$ from cells incubated in the presence of [^{14}C]pyruvate and [^{14}C]glucose was used as a measure of mitochondrial function. When compared to controls, cells isolated from the DSG-treated animals demonstrated a 73% decrease in CO_2 production when incubated with labeled pyruvate. There was no difference in lactate or pyruvate production, however. Finally, mean ATP levels were decreased by

40% relative to controls. Taken together, this data indicates a reduced mitochondrial respiratory capacity in the enterocytes of the DSG-treated animals. These results are tabulated in Table VI-6.

DISCUSSION

In normal rats, at the dose used, DSG induced a moderate 26% impairment of weight gain. This has been reported by others in rats and mice, although using two to four times higher doses (11,4,24,25), and in large animals using comparable doses (13,15,26). Interestingly, however, primates appear relatively unaffected, even at prolonged administration of 2-6 mg/kg/d (40).

The reduced weight gain could not be explained by any reduction in feed intake, which was comparable to controls. Some increased caloric utilization secondary to the hyperreactive behavior may be involved. Moreso, however, it may be the result of the observed decrease in fat absorption. Fat malabsorption with DSG has not been previously described in the literature. Absorption of fat is dependent on several processes (41). In these studies, fat malabsorption is unlikely to be due to any impairment of mechanical digestion since there was no effect on absorption of energy. The decrease in villus sagittal section area was small, and therefore it is unlikely to be entirely due to a change in mucosal surface area available for absorption. Impairment of hepatic function or biliary secretion of bile acids could be involved. However, although moderate increases in hepatic transaminase levels have been reported after 1 week of treatment with DSG, no effect on bilirubin levels have been observed (24). The significant increase in electrical conductance of the terminal ileum, as a reflection of increased permeability, suggests some disruption of the integrity of this region of the small intestine. However, 3OMeG uptake, as a reflection of enterocyte function, was not impaired. Further studies are needed to determine if reuptake of bile acids by the ileum may be affected, however, as this could result in a net decrease in the bile acid pool and therefore induce fat malabsorption.

Other adverse effects of DSG included severe impairments of the hematopoietic system, which is known to be the most significant toxicity of this agent (17,25). This has been shown to be secondary to a

suppression of proliferation and differentiation of immature bone marrow cells, *in vivo* and *in vitro* (2). Its effects, however, are cytostatic, not cytolytic, and is unlikely to be a serious disadvantage of DSG if used for short-term treatment only. Alopecia in rats has also been reported (25). Neurotoxicity has not been previously described with DSG, but may be reflected in the hyperreactive and aggressive behavior noted. Alternatively, it may be a reflection of otherwise difficult to diagnose gastrointestinal disturbances, which have been reported in dogs and humans (26,17). Finally, the mild diarrhea noted may be osmotic in nature, secondary to fat malabsorption.

With respect to small intestinal and enterocyte function, no significant changes in 3OMeG fluxes were observed in either the jejunum or ileum. Possible increased permeability of the ileum is suggested by the significant increase in *in vitro* electrical conductance and increased ^{99}Tc-DTPA recovery, *in vivo*. However, *in vivo* lactulose and mannitol recoveries were not altered. Further studies utilizing various probes *in vitro* need to be studied, to avoid confounding variables which can affect studies in intact animals, as discussed in preceding chapters (42).

The marked impairment of mitochondrial energy production by enterocytes also has not been previously described. One can speculate as to the widespread effects these alterations in energy metabolism may have on growth, animal well-being and small bowel function. Energy is required for most cellular processes, and this effect may explain some of the cytostatic actions of this agent on the hematopoietic system as well as maintenance of other cells undergoing rapid turnover such as epithelial cells (explaining the alopecia observed) and intestinal mucosa (reflected in the small but significant decrease in villus size throughout the bowel). In addition, the impaired weight gain may partly be a reflection of a global inability to maintain body cell mass. Finally, this may also account for the possible increase in bowel permeability, as the functional integrity of intracpithelial tight junctions appears to be strongly dependent on energy (43).

In summary, DSG, at the dose used, has significant adverse effects on growth and animal well-being in the normal rat. Fat absorption is significantly compromised, and may account for some of the impaired

weight gain observed. At the small bowel level, a slight increase in intestinal permeability may be induced in the ileum, but this effect is small and not associated with any significant effects on enterocyte function with respect to glucose uptake. Significant toxicities of the hematopoietic system need to be considered. However, as others have demonstrated these to be cytostatic in nature, they need not rule out the use of DSG as short term therapy, especially in view of its significant synergistic immunosuppressive effects with CsA. Finally, the alterations in mitochondrial energy production need to be further investigated, specifically looking at the reversibility of these effects and results with short-term therapy of DSG. Taken together, with little direct effect on small bowel function, our results suggest that DSG may be a potential candidate for use in SBT, likely in synergistic combination with another agent such as CsA. However, considerably more work must be done investigate its adverse effects on fat malabsorption and weight gain.

Table VI-1

Effects of Deoxyspergualin on Weight gain, Feed intake and Energy and Fat Absorption

	Weight Gain (g/d)	Feed Intake (g/kg/d)	% Energy Absorption	% Fat Absorption
Control (n=20)	3.15 ± 0.07	69.42 ± 1.11	81.3 ± 0.6	76.1 ± 0.9
Deoxyspergualin (n=10)	2.33 ± 0.09 ***26%↓** $p < 0.001$	69.75 ± 1.05 0%↔ $p = 0.739$	80.3 ± 1.4 1%↓ $p = 0.391$	66.9 ± 1.8 ***12%↓** $p < 0.001$

Values are means ± SEM.

***** significant difference compared to controls ($p<0.05$).

% values indicated relative to controls (↑, increase; ↓, decrease).

Table VI-2

Effects of Deoxyspergualin on Serum and Hematological Values

	Na^+ (mmol/l)	K^+ (mmol/l)	Creatinine (mmol/l)	Glucose (mg/dl)	WBC ($10^3c/mm^3$)	RBC ($10^3c/mm^3$)	HgB (g%)	Hct (%)
Control 2 (n=10)	143.2 ± 0.6	7.6 ± 0.8	0.42 ± 0.04	128.5 ± 0.5	7.8 ± 0.6	8.6 ± 0.2	16.4 ± 0.2	43.8 ± 0.9
Deoxyspergualin (n=10)	143.4 ± 0.6 0%↔ p=0.73	6.1 ± 0.7 19%↓ p=0.20	0.61 ± 0.05 45%↑ p=0.04	118.4 ± 17.7 8%↓ p=0.75	4.4 ± 0.8 ***43%↓** p=0.003	5.9 ± 0.3 ***32%↓** p<0.001	12.4 ± 0.4 ***25%↓** p<0.001	31.7 ± 1.4 ***28%↓** p<0.001

Values are means ± SEM.

* significant difference compared to controls ($p<0.05$); compared with student's t-test.

% values indicated relative to controls (↑, increase; ↓, decrease).

WBC, white blood cell count; RBC, red blood cell count; HgB, hemaglobin; Hct, hematocrit.

Table VI-3

Effects of Deoxyspergualin on *In Vivo* Intestinal Permeability

	^{99}Tc-DTPA	Mannitol	Lactulose	Lactulose/ Mannitol
Control 2 (n=10)	2.24 ± 0.18	1.97 ± 0.11	0.81 ± 0.04	0.42 ± 0.06
Deoxyspergualin (n=10)	3.71 ± 0.66 **66%↑** p=0.055	1.98 ± 0.13 1%↑ p=0.957	0.79 ± 0.08 2%↓ p=0.121	0.41 ± 0.04 2%↓ p=0.448

Data is presented as percent recovery of orally administered marker in urine; mean ± SEM.

* significant difference compared to controls ($p<0.05$).

% values indicated relative to controls (↑, increase; ↓, decrease).

Table VI-4
Effects of Deoxyspergualin on Villus Morphometry and Density

	Width ($\mu m \times 10^2$)	Height ($\mu m \times 10^2$)	Area ($\mu m^2 \times 10^2$)	# Villi per 20X field
JEJUNUM				
Control 2 (n=10)	2.00 ± 0.05	6.86 ± 0.07	8.03 ± 0.21	5.41 ± 0.19
Deoxyspergualin (n=10)	1.97 ± 0.06 1%↓ p=0.773	6.64 ± 0.11 3%↓ p=0.094	6.95 ± 0.21 ***13%↓** p=0.001	5.53 ± 0.16 2%↑ p=0.693
ILEUM				
Control 2 (n=10)	2.06 ± 0.06	4.07 ± 0.07	4.94 ± 0.17	4.65 ± 0.33
Deoxyspergualin (n=10)	1.90 ± 0.08 8%↓ p=0.059	3.93 ± 0.07 3%↓ p=0.269	4.22 ± 0.17 ***16%↓** p=0.007	5.54 ± 0.26 19%↑ p=0.106

Data is presented as villus width, height from lamina propria and saggital section area; expressed as mean ± SEM.
***** significant difference compared to controls ($p<0.05$); compared with student's t-test.
% values indicated relative to controls (↑, increase; ↓, decrease).

Table VI-5
Effects of Deoxyspergualin on *In Vitro* 3OMeG fluxes and Electrophysiological Parameters

	J_{ms}	J_{sm}	J_{net}	PD	I_{sc}	G
JEJUNUM						
Control (n=20)	1.24 ± 0.08	0.84 ± 0.06	0.41 ± 0.05	1.70 ± 0.12	39.29 ± 3.16	22.56 ± 1.54
Deoxyspergualin (n=10)	1.31 ± 0.06 9%↓ p=0.317	0.83 ± 0.10 1%↓ p=0.736	0.29 ± 0.08 29%↓ p=0.147	1.47 ± 0.35 14%↓ p=0.424	32.17 ± 2.32 18%↓ p=0.117	23.73 ± 2.61 5%↑ p=0.68
ILEUM						
Control (n=20)	1.19 ± 0.08	0.82 ± 0.08	0.36 ± 0.06	2.26 ± 0.15	40.86 ± 2.59	18.25 ± 1.50
Deoxyspergualin (n=10)	1.33 ± 0.12 12%↑ p=0.403	0.87 ± 0.11 6%↑ p=0.676	0.46 ± 0.11 28%↑ p=0.382	2.70 ± 0.37 19%↑ p=0.625	45.42 ± 6.84 11%↑ p=0.916	24.19 ± 1.80 ***33%↑** p=0.019

Values are means ± SEM. J_{ms}, flux from mucosa to serosa; J_{sm}, flux from serosa to mucosa; J_{net}, numerical difference between J_{ms} and J_{sm} in µmol/cm²/hr; PD, potential difference in mV; Isc, short-circuit current in µAmp/cm²/hr; G, conductance in mS/cm².

*significant difference compared to controls ($p<0.05$); compared with student's t-test; % values indicated relative to controls (↑, increase; ↓, decrease).

Table VI-6

Effects of Deoxyspergualin on *In Vitro* Mitochondrial Energy Production

	[^{14}C]Glucose utilization (ng/hr/mg protein)	[^{14}C]Pyruvate utilization (nmol/hr/mg)	Lactate production (mg/hr/mg protein)	Pyruvate production (mg/hr/mg protein)	ATP production (nmol/μg protein)
Control (n=4)	24.20 ± 1.21	1.19 ± 0.15	0.10 ± 0.02	0.0067 ± 0.0006	1.32 ± 0.12
Deoxyspergualin (n=4)	9.55 ± 2.13 ***60%↓** p<0.001	0.32 ± 0.14 ***73%↓** p<0.001	0.10 ± 0.01 0%↔ p=0.97	0.0078 ± 0.0011 8%↑ p=0.48	0.80 ± 0.14 ***39%↓** p=0.029

Values are means ± SEM.

***** significant difference compared to controls ($p<0.05$); compared with student's t-test.

% values indicated relative to controls (↑, increase; ↓, decrease).

REFERENCES

1. Takeuchi T, Iinuma H, Kunimoto S.Masuda T, Ishizuka M, Takiuchi M. A new antitumor antibiotic, spergualin. J Antibiot 1981; 34: 1619-21.

2. Nishimura K, Tokunaga T. Effects of 15-Deoxyspergualin on the induction of cytotoxic T lymphocytes and bone marrow suppression. Transplant Proc 1989; 21(1): 1104-7.

3. Jiang H, Takahara S, Takano Y, Machida M, Iwasaki A, Kokado Y, *et al.* In vitro immunosuppressive effect of Deoxymethylspergualin. Transplant Proc 1990; 22(4): 1633-7.

4. Bumgardner GL, Roberts JP. New immunosuppressive agents. Advances in Liver Transplant 1993; 22(2): 421-9.

5. Auchincloss H, Jr., Sachs DH. Transplantation and graft rejection. In: Paul WE, editor. Fundamental Immunology, third edition. New York: Raven Press, 1993: 1099-141

6. Gannedahl G, Karlsson-Parra A, Totterman TH, Tufveson G. 15-Deoxyspergualin inhibits antibody production in mouse to rat heart transplantation. Transplant Proc 1993; 25(1): 778-80.

7. Suzuki S, Kanashiro M, Watanabe H, Sakakiraba I, Amemiya H. Therapeutic effects of 15-Deoxyspergualin on acute graft rejection detected by ^{31}P nuclear magnetic resonance spectrography, and its effect on rat heart transplantation. Transplantation 1988; 46(5): 669-72.

8. Suzuki S, Kanashiro M, Watanabe H, Sakakiraba I, Amemiya H. Effect of 15-Deoxyspergualin on rejection detected by ^{31}P nuclear magnetic resonance spectroscopy and in vivo mechanism of action in rat heart transplantation. Transplant Proc 1989; 21(1): 1094-5.

9. Yuh DD, Morris RE. 15-Deoxyspergualin is a more potent and effective immunosupr ant than Cyclosporine but does not effectively suppress lymphoproliferation in vivo. Transpla ; 23(1): 525-9.

10. Schubert G, Stoffregen C, Looke G, Timmermann W, Schung T, Thiede A. Synergistic effect of 15-deoxyspergualin and cyclosporin A in pancreatic transplantation. Transplant Proc 1989; 21(1): 1096.

11. Kamata K, Okubo M, Masaki Y, Inoue K, Umetani N, Sato N, Uchida H. Effect of 15-deoxyspergualin on the survival of thyroid allografts in mice. Transplant Proc 1989; 21(1): 1099-103.

12. Von Specht B-U, Dibelius A, Konigsberger H, Lodde G, Roth H, Permanetter W. Prolongation of islet and pancreas graft survival in spontaneous diabetic BB/W rats by perioperative cyclosporin or 15-deoxyspergualin therapy. Transplant Proc 1989; 21(1): 965-7.

13. Koyama I, Kadokura M, Hoshino T, Omoto R. Effective use of 15-deoxyspergualin in kidney transplantation. Transplant Proc 1989; 21(1): 1088-9.

14. Niiya S, Suzuki S, Hayashi R, Watanabe H, Itoh J, Amemiya H. 15-deoxyspergualin: a powerful rescue drug for rejection in canine kidney grafting. Transplant Proc 1989; 21(1): 1076.

15. Morisaki F, Sakagami K, Orita K. Effect of 15-deoxyspergualin on class II antigen expression in hepatic sinusoidal lining cells and on infiltration of monomuclear cells in swine liver transplantation. Transplant Proc 1989; 21(1): 1077-80.

16. Okubo M, Tamura K, Kamata K, Tsukamoto Y, Nakayama T, Osakabe T, *et al.* 15-Deoxyspergualin "rescue therapy" for methylprednisolone-resistant rejection of renal transplants as compared with anti-T cell monoclonal antibody (OKT3). Transplantation 1993; 55(3): 505-8.

17. Amemiya H, Taguchi Y, Fukao K, Isono K, Omoto R, Ota K, *et al.* Establishment of rejection therapy with Deoxyspergualin by multicentral controlled clinical studies in renal recipients. Transplant Proc 1993; 25(1): 730-3.

18. Takahara S, Moutabarrik A, Kokado Y, Kameoka H, Takano Y, Zaid D, et al. Use of 15-Deoxyspergualin in treatment of acute rejection in kidney transplant patients. Transplant Proc 1993; 25(3): 2254-8.

19. Gores PF, Najarian JS, Stephanian E, Lloveras J, Kelley S, Sutherland DER. Insulin independence in type I diabetes after transplantation of unpurified islets from single donor with 15-Deoxyspergualin. Lancet 1993; 341: 19-21.

20. Gannedahl G, Wanders A, Carlsson M, Tufveson G. 15-Deoxyspergualin effects in rat heart allograft transplantation--Relation to dose, timing, and cyclosporine. Transplantation 1993; 55(2): 455-6.

21. Gannedahl G, Wanders A, Carlsson M, Tufveson G. 15-Deoxyspergualin potentiates the immunosuppressive effect of Cyclosporine A in a rat heart allograft model. Transplant Proc 1993; 25(1): 768-9.

22. Tanaka S, Okada H, Uda K, Fujioka M, Makabe M, Orita T, Orita K. Mechanism of graft-versus-host disease and inhibition with deoxyspergualin on small bowel transplantation in rats. Transplant Proc 1992; 24(3): 1161-3.

23. Andreoni B, Biffi R, Derai P, Bardella MT, Velio P, Pozzi S, *et al.* In: Abstracts of the IIIrd International Symposium on Small Bowel transplantation; 1993 Nov; Paris: P90.

24. Jindal RM, Soltys K, Yost F, Beer E, Tepper MA, Cho SI. Effect of Deoxyspergualin on the endocrine fucntion of the rat pancreas. Transplantation 1993; 56(5): 1275-7.

25. Yuh DD, Morris RE. The immunopharmacology of immunosuppression by 15-Deoxyspergualin. Transplantation 1993; 55(3): 578-91.

26. Amemiya H, Suzuki S, Niiya S, Fukao K, Yamanaka N, Ito J. A new immunosuppressive agent, 15-deoxyspergualin in dog renal allografting. Transplant Proc 1989; 21: 3468-70.

27. Canadian Council on Animal Care. Guide to the care and use of Experimental Animals. The Council, 1980.

28. Albert DJ, Richmond SE. Septal hyperreactivity: A comparison of lesions within and adjacent to the septum. Physiol & Behav 1975; 15: 339-47.

29. Association of Official Analytical Chemist (1986): Methods of analysis (23 Ed.). AOAC, Washington, D.C.

30. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1956; 226: 497-509.

31. Elia M, Behrens R, Northrop C, Wraight P, Neale G. Evalation of mannitol, lactulose and ^{51}Cr-labelled ethylenediaminetetra-acetate as markers of intestinal permeability in man. Clin Sci 1987; 73: 197-204.

32. Hamilton I, Rothwell J, Archer D, Axon ATR. Permeability of the rat small intestine to carbohydrate probe molecules. Clin Sci 1987; 73: 189-96.

33. Joyce Loebl. Image analysis: Principles and practice. Great Britain: Joyce Loebl, 1985.

34. Erickson RA, Rivera N. Effect of difluoromethylornithine (DFMO) on NSAID-induced intestinal injury in rats. Dig Dis Sci 1992; 37(12): 1833-9.

35. Pahl MV, Erickson RA, Vaziri ND, Khamisch G, Oveisi F, Jan K, *et al.* Intestinal morphometry and bile acid-induced mucosal injury in chronic experimental renal failure. J Lab Clin Med 1990; 115: 572-8.

36. Field M, Fromm D, McColl I. Ion transport in rabbit ileal mucosa. I. Na and Cl fluxes and short-circuit current. Am J Physiol 1971; 220(5): 1388-96.

37. Fedorak RN, Chang EB, Madara JL, Field M. Intestinal adaptation to diabetes: Altered Na-dependent nutrient absorption in streptozocin-treated chronically diabetic rats. J Clin Invest 1987; 79: 1571-8.

38. Madsen KL, Yanchar NL, Fedorak RN, Sigalet DL. Effect of FK506 and Deoxyspergualin on intestinal metabolic function. submitted.

39. Roediger WE, Truelove SC. Method of preparing isolated colonic epithelial cells (colonocytes) for metabolic studies. Gut 1979; 20: 484-8.

40. Fukao K, Iwasaki H, Yuzawa K, Otsuka M, Yu Y, Sharma N, *et al.* Immunosuppressive effect and toxicity of 15-Deoxyspergualin in cynanolgus monkey. Transplant Proc 1991; 23(1): 556-8.

41. Thomson ABR, Fedorak RN. The small intestine. In: Thomson ABR, Shaffer EA, editors. First principles of gastroenterology: The basis of disease and an approach to management. Toronto: Canadian Association fo Gastroenterology, 1992, 179-275.

42. Peters TJ, Bjarnason I. Uses and abuses of intestinhal permeability measurements. J Gastroenterol 1988; 2(3): 127-32.

43. Mandel LJ, Bacallao R, Zampighi G. Uncoupling of the molecular 'fence' and paracellular 'gate' functions in epithelial tight junctions. Nature 1993; 361: 552-5.

CHAPTER VII

RS61443

INTRODUCTION

RS61443 (mycophenolate mofetil) is a semisynthetic prodrug, a derivative of the fungal antibiotic mycophenolic acid (MPA), isolated from the mold *Penicillin glaucum* (1). Its mode of immunosuppressive action appears to lie in its ability to selectively depress *de novo* purine synthesis in lymphocytes, subsequently inhibiting their proliferation and differentiation (1-6). The depletion of purine nucleotide pools also depresses the production of adhesion molecules required for the interaction of lymphocyte with their target cells and other components of the immune rejection response (7, 8).

With its markedly different mechanism of action compared to other immunosuppressives, RS61443 is extremely efficacious in complementing or enhancing the actions of other agents within drug combination protocols. It has been shown that 20 mg/kg/d, p.o. in combination with cyclosporin A (CsA) and steroids or with brequinar sodium can prolong the survival of rat heart allografts (2, 9). Similar doses of RS61443 and CsA markedly prolong survival of canine liver allografts with minimal side effects (10). In human trials, oral doses of 100-3500 mg/d have been successfully utilized in place of azathioprine for induction and prophylaxis immunosuppression in renal transplant recipients also receiving CsA, with markedly less toxicity than occurs with azathioprine (11). Similar efficacy is seen with RS61443's use as "rescue" therapy in treating rejection of human cardiac, liver and renal transplants (12-14).

The efficacy of RS61443 in other organ transplantation models led to its investigation as a promising agent to be used in SBT. When used alone, prolonged administration of 25 mg/kg/d, i.p. successfully prevents graft-versus-host-disease (GVHD) but not host-versus-graft (HVG) rejection in a semiallogeneic rat SBT model (15). Similar results have been found by others (16). When administered with therapeutic

doses of CsA and prednisone, however, RS61443 at 20 mg/kg/d markedly prolongs survival of fully allogeneic canine segmental small bowel allografts, whereas either drug alone is ineffective (17). The combined action appears to be complementary rather than synergistic, as use of RS61443 with subtherapeutic doses of CsA or FK506 does not lend any allograft survival advantage, although the recipients have a better clinical course, with better eating and weight gain patterns than with CsA or FK506 alone (17,18). This differs from the results seen in rat cardiac transplantation, where RS61443 and subtherapeutic doses of CsA successfully prolong allograft survival (2). This may reflect the greater immunogenicity of small bowel as opposed to cardiac allografts.

Some investigators feel, therefore, that higher doses of RS61443 are required for SBT. However, serious dose-dependent toxic effects have become associated with this agent. These primarily involve the gastrointestinal tract and include nausea, vomiting, diarrhea, gastritis and anorexia, as seen in renal transplanted dogs receiving doses of 40 mg/kg/d. In humans, higher doses may occasionally produce hemorrhagic gastritis or other gastrointestinal symptoms such as nausea, vomiting and diarrhea, sometimes requiring cessation of the drug (11,12,14). Implications of these effects on the nutritional capacity of the bowel have not been reported. However, they suggest the possibility that RS61443 may further jeopardize the nutritional and absorptive function of an already compromised small bowel allograft. Therefore, before being seriously considered for use in SBT, these effects of RS61443 on the bowel, especially as they relate to the nutritional and absorptive capacity of the intestine, must be further defined and understood.

MATERIALS AND METHODS

ANIMALS

Juvenile male Lewis rats (275-300 g) were obtained from Charles River Canada, St. Constant, P.Q., and housed in individual plexiglass cages with free access to water and standard lab rat chow (Lab Diet,

PMI Feeds, St. Louis, MO.) with an approximate composition of 23.0% protein, 4.5% fat, 6.0% fiber, 8.0% ash and 2.5% minerals. Day/night cycles were 12 hours and the temperature was maintained at 20 ± 2°C. Feed intake was monitored weekly, and body weight changes, biweekly. Animal care was in accordance with the guidelines of the Canadian Council of Animal Welfare (19). The experimental protocol was approved by the Animal Welfare Committee of the University of Alberta

EXPERIMENTAL GROUPS

RS61443 was suspended in carboxymethlycellulose (CMC) and administered to the test animals (n=10) at a dose of 25 mg/kg/d, injected subcutaneously (s.c.) into the nape of the neck for 6 days, followed by the same dose on alternate days for 5 more weeks. Control animals received a corresponding volume/kg of the drug vehicle, s.c., on the same schedule. As discussed in the "Introduction" of this thesis, the complete study undertaken was performed in two sets of experiments. This subsequently provided two control groups against which the test group could be compared: "control 1" (n=10) which was studied alongside RS61443 in the first set of experiments, and an overall control group of n=20, consisting of "control 1" and "control 2", the latter (n=10) utilized during the second set of experiments. Each control group was divided in half, with 5 animals receiving CMC, and 5 receiving MCT oil, the vehicle used with the CsA studies.

DRUGS

RS61443 (a generous gift of Bristol-Myers Squibb, Seattle, Washington) was freshly prepared each day before injecting. An appropriate weight was first dissolved in a defined volume of 95% ethanol using a 40°C water bath, and then suspended in CMC (Sigma, St. Louis, MO.) (final vehicle composition of 9:1 CMC:ethanol) by sonication for 2 minutes, with a final concentration of 30 mg/ml. Drug concentrations were prepared so that equivalent volumes (0.83 ml/kg) were injected. Control animal also received equivalent volumes per kg of the liquid vehicles.

DRUG MONITORING

Whole blood RS61443 levels were drawn after two weeks of drug administration by internal jugular vein puncture and at sacrifice by direct cardiac puncture. Both peak (2 hours post-dose) and trough (24 hours post-dose) levels were obtained. Blood was drawn into tubes containig anticoagulant, and analyzed by high performance liquid chromatography (HPLC) as described previously (20).

IN VIVO NUTRIENT BALANCE STUDIES

After 33 days of treatment, the animals were placed in individual metabolic cages, preconditioned for 3 days, and then underwent a 3 day balance study with daily measurement of food-intake and fecal collection. Standard lab rat chow was given *ad lib*, with a composition as described above. Total carbohydrate and fat content of feed and feces were determined using standard methods (21,22). Briefly, feed and collected feces were completely desiccated in a freeze-dryer, and the percentage water determined directly by the change in total weight. Dried feces were then ground into a powder, and 0.2 g of sample used to determine the total energy content using a Gallenkamp Ballistic Bomb Calorimeter (London, England). 0.25 g of sample was then rehydrogenated and the fat extracted into the solvent layer of a 0.9% sodium-chloride-chloroform:methanol (2:1) extraction mixture. After a second extraction of the aqueous layer, to ensure completeness, the solvent was evaporated off and the final contents weighed to determine the amount of total fat extracted. From these results, the percentages of energy and fat absorption from feed intake were calculated directly.

IN VIVO INTESTINAL PERMEABILITY

In the fifth week of the study, animals were fasted overnight and then gavaged with 100 mg of mannitol and 100 mg of lactulose in 2 ml of water during which time the animals were allowed free access to water but not food. Urine was quantitatively collected and urinary recovery of each compound was measured using high performance liquid chromatography and reported as both the percentage recovery of the total oral dose of each carbohydrate probe, and the ratio of lactulose to mannitol recovered. This latter measurement is felt to best reflect the permeability of the bowel per unit surface area (23,24).

VILLUS MORPHOMETRY AND DENSITY

At sacrifice, sections of jejunum and ileum from control and test animals were fixed in formalin and mounted in paraffin blocks. They were cut so that both longitudinal and cross-sections of bowel were obtained which were then stained with haematoxylin and eosin. As an indirect measurement of mucosal surface characteristics, sections of ileum and jejunum, for each group, were photomicrographed at a magnification of 100X. Morphometric analyses were performed using an Image analysis system (Joyce Loebl, Magiscan) (25). Image analysis systems have been used to efficiently determine morphometric parameters of large numbers of intestinal villi in several recent studies analyzing intestinal mucosal architecture (26,27). Parameters studied included villus length, as measured from the lamina propria, width and overall sagittal section area for 25 villi per histological section. Three sections from both the jejunum and ileum for each test group and four sections from both the jejunum and ileum of the controls were analyzed, resulting in 75 to 100 villi being measured per group for each region of small bowel. The number of villi per microscopic field (at 20X magnification) were determined as a measure of villus density.

IN VITRO GLUCOSE FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

After 6 weeks of treatment, animals were sacrificed with an intraperitoneal pentobarbital overdose and *in vitro* small bowel function studies were performed. Segments of distal ileum and proximal jejunum, taken approximately 2 to 3 cm from the ileocecal valve and ligament of Trietz, respectively, were quickly excised, rinsed with ice-cold normal Ringers solution, and stored in ice-cold Ringers gassed with carbogen (5% CO_2 in O_2) during the preparation for mounting onto Ussing chambers as previously described (28,29). Briefly, the intestine was split along the mesenteric border and segments of approximately 2 cm were then quickly stripped of the serosa and underlying muscle layers and then clamped into Ussing chambers . This subsequently divides the chamber into mucosal and serosal compartments. Tissues were bathed on both sides by normal Ringers (Na^+, 143 mM; K^+, 5 mM; Ca^{2+}, 1.25 mM; Mg^{2+} 1.1 mM; Cl^-, 123.7 mM; HCO_3, 25 mM; $HPO_4 + H_2PO_4$, 1.95 mM) with 20 mM fructose,

and gassed with carbogen to pH of 7.4. A circulating water bath maintained the chamber temperature at 37°C. For measurement of unidirectional fluxes, 3-O-methyl-D-glucose (3OMeG) was present at a concentration of 20 mM on both mucosal and serosal sides and 5 μCi of [^{3}H]-3OMeG was added to either the mucosal or serosal side of the tissue. To ensure steady-state conditions, tissues were maintained under these conditions for 20 minutes. Previous studies have shown that glucose flux is constant over this time period (28). Unidirectional mucosal-to-serosal (J_{ms}) and serosal-to-mucosal (J_{sm}) fluxes were measured in paired tissues by collection of aliquots of incubation solution from each side of the chambers and determining the radioactivity in a Beckman LS5801 β-Scintillation Counter (California). Tissue pairs were discarded if electrical conductance (see below) varied by >15%. Four consecutive 5 minute fluxes were determined. Tissue response to 5 mM theophylline was used to confirm viability at the completion of the experiment.

Electrophysiological parameters were monitored throughout. The spontaneous transepithelial electrical potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (I_{sc}) with an automatic voltage clamp (DCV 1000 World Precision Instruments, New Haven, CT), except for 3-5 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue conductance (G) was calculated from potential difference and I_{sc} according to Ohm's law, where voltage (V) = current (I_{sc}) x resistance (R) and conductance (G) is the reciprocal of R (28).

STATISTICAL ANALYSES

Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using a computerized statistical package (SigmaStat Statistical Analysis System, Jandel Corporation). Means between the two groups (controls vs treatment) were compared using the Student's t-test unless the distribution was nonparametric (as determined directly by the program, using the Kolmogorov-Smirnov test of normality, with a p value of >0.05) in which case a distribution-free test comparing the medians is more appropriate, and was performed with a Mann-Whitney rank sum test. A p-value of <0.05 was

considered statistically significant. No statistically significant differences were determined between the MCT and CMC treatments, and in all but three sets of results (stool water content, lactulose/mannitol permeability and villus morphometry), no differences were found between the two control groups ("control 1" and "control 2"). Subsequently, all control animals were combined into one control group with n=20. In studies where the two significantly differed , the differences were assumed to be secondary to altered laboratory processing of the samples, and the RS61443 group was compared to its respective "control 1" group, only.

RESULTS

WEIGHT GAIN, FEED INTAKE & *IN VIVO* NUTRITIONAL BALANCE STUDIES

Body weight gain over the six week period was 12% less than controls, but this did not reach statistical significance. Feed intake, and in vivo absorption of fat were unchanged. Energy (kcal) absorption was increased by 2%, and although this was statistically significant, the increment is extremely small. These effects are tabulated in Table VII-1.

OTHER EFFECTS

Animals appeared well and active throughout the study. The animals developed mildly inspissated stools, however, with stool water content of 23.9 ± 1.5% which was 22% less than quantitated in the control animals (30.6 ± 2.4%). This was statistically significant (p=0.04).

DRUG LEVELS

Mean peak and trough levels obtained after two weeks of drug administration were 5113 ± 165 ng/ml and 1591 ± 391 ng/ml, respectively. At sacrifice, the peak and trough levels were 4034 ± 590 ng/ml and 1177 ± 262 ng/ml, respectively. The differences between levels at the different time periods were not statistically significant (p=0.326 between peak levels and p=0.333 between trough levels). Mean overall peak and trough levels were thus 4342 ± 454 ng/ml and 1384 ± 207 ng/ml, respectively.

VILLUS MORPHOMETRY AND DENSITY

Significant increases in villus width, height, and sagittal section area were observed in the jejunum of the treated animals, compared to controls, as seen in Table VII-3 and Plate 2 (Appendix I). This was associated with a 32% reduction in number of villi per 20X magnification field. Villus size and density were unchanged in the ileum, however.

IN VIVO BOWEL PERMEABILITY

RS61443-treated animals demonstrated a significant 36% decrease in mannitol recovery relative to controls. However, because of a 26% decrease (not significant) in lactulose recovery, the lactulose/mannitol ratio was comparable to the controls (Table VII-2).

IN VITRO 3OMeG FLUXES AND ELECTROPHYSIOLOGICAL PARAMETERS

The effects of RS61443 on *in vitro* 3OMeG transmural fluxes are summarized in Table VII-4. In the jejunum, a 25% decrease in J_{ms} resulted in a 80% decrease of J_{net}. This corresponded with a 32% decrease in I_{sc}. A reversal of these effects were seen in the ileum, where J_{ms} was increased by 34% accompanied by a 2-fold increase in J_{net} and a 51% increase in I_{sc}. There were no significant changes in J_{sm} or electrical conductance as compared to controls.

DISCUSSION

The optimal dose of RS61443 for SBT in the rat model has not been determined. Previous studies have utilized doses ranging from 20-30 mg/kg/d, p.o. (16-18), to 25 mg/kg/d, i.p (15). In this study, the drug was administered i.m., to more closely simulate a post-operative regimen where the small bowel may not reliably absorb an oral dosage. As with most agents, whole blood levels of the drug are perhaps more important than actual dosages, in order to achieve consistent therapeutic levels and avoid toxicity. Whole blood peak and trough levels in the treated rats averaged 4342 ± 454 μg/L and 1384 ± 413μg/L, respectively. Although the peak levels are approximately two-fold greater than those obtained by D'Alessandro *et al* in successful canine heterotopic small bowel transplantation (17) and the desired

trough levels have not been reported extensively in the literature, we did not note any significant toxicity's in the test animals. Indeed, RS61443 induced no significant adverse effects on growth or well-being in the test animals. Nutrient absorption was also virtually unchanged compared to controls.

At the level of the small bowel, a significant increase in villus size was observed in the jejunum. However, villus density was decreased. If it is assumed that a villus is a cylinder, the mean surface area can be calculated using the equation: $\pi r^2 + 2\pi rh$, where r is the radius and equivalent to one half of the measured width and h is the height. Using this equation, the mean surface area of the control 1 group equals 26.9 x 104 μm^2 and that of the RS61443 animals equals 37.8 x 104 μm^2. This latter value represents a 41% increase in mean surface area. Since the villus density in this region of the small bowel decreased by 32% relative to the control group, it can be assumed that the overall mucosal surface area in the sections of jejunum analyzed was not greatly different between the test and control animals. Of course, however, intestinal villi are not absolute cylinders and these calculations cannot precisely determine their true surface area (30). However, they provide an estimate and indicate that the proportional increase in villus surface area may, at the least, approximate the decrease in density, such that the conclusion remains the same. These effects of RS61443 on mucosal architecture have not been previously described, and the mechanism by which this occurs is elusive.

The integrity of the bowel, as reflected by permeability studies with *in vivo* probes and *in vitro* electrical conductance calculations was also unchanged in the treated animals. Of note, however, was the significant decrease in forward mucosal-to-serosal flux of 3OMeG in the jejunum. The explanation for this can only be speculated as such an effect has not been previously reported in the literature. It may be that the morphological increase in villus size was not associated with a proportional increase in glucose transporters. Subsequently, the decrease in villus density may have resulted in a net reduction of glucose transporters available. Studies to assess this directly, such as the determination of [^{3}H]phlorizin binding to glucose transporter sites may clarify this (31). No changes in carbohydrate absorption occurred *in vivo*,

however, likely because of functional adaptation by the ileum, where equivalent increases in mucosal-to-serosal and net 3OMeG fluxes were observed.

In summary, RS61443, at the dose use, had little effect on growth, animal well-being and nutrient absorption in normal rats and is likely safe to use in SBT. Its effect on the jejunum in decreasing active 3OMeG uptake has not been previously described and is difficult to explain. Its association with the observed altered villus morphometry and density, however, favors an explanation based on decreased density of glucose carriers. This may be a region-specific effect. Therefore, although efficacious in animal models of small bowel transplantation, further studies on these functional effects of RS61443 on nutrient absorption need to be done, especially if considering this agent for use in transplantation of segmental allografts of the jejunum, as could be performed with living-related donors.

Table VII-1

Effects of RS61443 on Weight gain, Feed intake and Energy and Fat Absorption

	Weight Gain (g/d)	Feed Intake (g/kg/d)	% Energy Absorption	% Fat Absorption
Control (n=20)	3.15 ± 0.07	69.42 ± 1.11	81.3 ± 0.6	76.1 ± 0.9
RS61443 (n=10)	2.76 ± 0.21 12%↓ p = 0.172	70.90 ± 1.00 2%↑ p = 0.297	83.1 ± 0.5 ***2%↑** p = 0.011	76.1 ± 0.6 0%↔ p = 0.965

Values are means ± SEM

***** significant difference compared to controls ($p<0.05$).

% values indicated relative to controls (↑, increase; ↓, decrease).

Table VII-2

Effects of RS61443 on *In Vivo* Intestinal Permeability

	Mannitol	Lactulose	Lactulose/ Mannitol
Control 1 (n=10)	3.77 ± 0.22	1.40 ± 0.11	0.41 ± 0.03
RS61443 (n=10)	2.42 ± 0.33 ***36%↓** p=0.005	1.03 ± 0.15 26%↓ p=0.086	0.43 ± 0.02 3%↑ p=0.197

Data presented as percent recovery of orally administered marker in urine; mean ± SEM.
* significant difference compared to controls ($p<0.05$); compared with student's t-test.
% values indicated relative to controls (↑, increase; ↓, decrease).

Table VII-3
Effects of RS61443 on Villus Morphometry and Density

	Width ($\mu m \times 10^2$)	Height ($\mu m \times 10^2$)	Area ($\mu m^2 \times 10^2$)	# Villi per 20X field
JEJUNUM				
Control 1 (n=10)	1.66 ± 0.05	4.74 ± 0.12	4.67 ± 0.19	6.58 ± 0.52
RS61443 (n=10)	1.82 ± 0.05 ***9%↑** p=0.004	6.16 ± 0.06 ***30%↑** p<0.001	6.56 ± 0.17 ***40%↑** p<0.001	4.46 ± 0.25 ***32%↓** p=0.003
ILEUM				
Control 1 (n=10)	1.70 ± 0.06	3.84 ± 0.14	4.13 ± 0.23	5.26 ± 0.41
RS61443 (n=10)	1.70 ± 0.07 0%↔ p=0.954	3.74 ± 0.06 3%↓ p=0.676	3.84 ± 0.15 7%↓ p=0.599	4.75 ± 0.49 10%↓ p=0.483

Data is presented as villus width, height from lamina propria and saggital section area; expressed as mean ± SEM.
* significant difference compared to controls ($p<0.05$); compared with student's t-test; % values indicated relative to controls (↑, increase; ↓, decrease).

Table VII-4
Effects of RS61443 on *In Vitro* 3OMeG fluxes and Electrophysiological Parameters

	J_{ms}	J_{sm}	J_{net}	PD	I_{sc}	G
JEJUNUM						
Control (n=20)	1.24 ± 0.08	0.84 ± 0.06	0.41 ± 0.05	1.70 ± 0.12	39.29 ± 3.16	22.56 ± 1.54
RS61443 (n=10)	0.93 ± 0.11 ***25%↓** p≤0.050	0.85 ± 0.09 1%↑ p=0.913	0.08 ± 0.08 ***80%↓** p=0.001	1.73 ± 0.37 2%↑ p=0.939	26.70 ± 3.24 ***32%↓** p=0.019	22.28 ± 2.02 1%↓ p=0.930
ILEUM						
Control (n=20)	1.19 ± 0.08	0.82 ± 0.08	0.36 ± 0.06	2.26 ± 0.15	40.86 ± 2.59	18.25 ± 1.50
RS61443 (n=10)	1.59 ± 0.05 ***34%↑** p=0.018	0.87 ± 0.08 6%↑ p=0.732	0.72 ± 0.10 ***100%↑** p=0.006	2.43 ± 0.01 8%↑ p=0.229	61.74 ± 3.85 ***51%↑** p<0.001	21.95 ± 1.37 20%↑ p=0.171

Values are means ± SEM. J_{ms}, flux from mucosa to serosa; J_{sm}, flux from serosa to mucosa; J_{net}, numerical difference between J_{ms} and J_{sm} in μmol/cm²/hr; PD, potential difference in mV; Isc, short-circuit current in μAmp/cm²/hr; G, conductance in mS/cm².

*significant difference compared to controls ($p<0.05$); compared with student's t-test; % values indicated relative to controls (↑, increase; ↓, decrease).

REFERENCES

1. Sweeney MJ, Hoffman DH, Esterman MA. Metabolism and biochemistry of mycophenolic acid. Cancer Res 1972; 32: 1803-9.

2. Platz KP, Sollinger HW, Hullett DA, Eckhoff DE, Eugui EM, Allison AC. RS-61443 -- a new, potent immunosuppressive agent. Transplantation 1991; 51(1): 27-31.

3. Burlingham WJ, Grailer AP, Hullett DA, Sollinger HW. Inhibition of both MLC and in vitro IgG memory response to tetanus toxoid by RS-61443. Transplantation 1991; 51(2): 545-7.

4. Morris RE, Hoyt EG, Murphy MP, Eugui EM, Allison AC. Mycophenolic acid morpholinoethylester (RS-61443) is a new imminosuppressant that prevents and halts heart allograft rejection by selective inhibition of T- and B-cell purine synthesis. Transplant Proc 1990; 22(4): 1659-62.

5. Allison AC, Eugui EM. Inhibitors of de novo purine and pyrimidine synthesis as immunosuppressive drugs. Transplant Proc 1993; 25(3)(supp 2): 8-18.

6. Franklin TJ, Cook JM. The inhibition of nucleic acid synthesis by mycophenolic acid. Biochem J 1969; 113: 515-24.

7. Allison AC, Kowalski W, Muller C, Waters R, Eugui EM. Mycophenolic Acid and Brequinar, inhibitors of purine and pyrimidine synthesis, block the glycosylation of adhesion molecules. Transplant Proc 1993; 25(3)(supp 2): 67-70.

8. Isobe M, Yagita H, Okumura K, Ihara A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. Science 1992; 255: 1125-7.

9. Kawamura T, Hullett D, Suzuki U, Bechstein W, Allison A, Sollinger HW. Enhancement of allograft survival by combination RS-61443 and DUP-785 therapy. Transplantation 1993; 55(4): 691-5.

10. Bechstein WO, Schilling M, Steele D, Hullet D, Sollinger HW. RS61443/cyclosporine combination therapy prolongs canine liver allograft survival. Transplant Proc 1003; 25(1), 702-3.

11. Deierhoi MH, Sollinger H, Deithelm A, Belzer F, Kauffman RS. One-year follow-up results of a phase I trial of Mycophenolate Mofetil (RS61443) in cadaveric renal transplantation. Transplant Proc 1993; 25(1): 693-4.

12. Sollinger H, Belzer FO, Deierhoe M, Diethelm A, Gonwa TA, Kauffman RS, *et al.* RS-61443: rescue therapy in refractory kidney transplant rejection. Transplant Proc 1993; 25(1): 698-9.

13. Klintmalm GB, Ascher NL, Busuttil RW, Deierhoi M, Gonwa TA, Kauffman R, *et al.* RS-61443 for treatment-resistant human liver rejection. Transplant Proc 1993; 25(1): 697.

14. Ensley RD, Bristow M, Olsen S, Taylor D, Hammond E, O'Connell J, *et al.* The use of Mycophenolate Mofetil (RS-61443) in human heart transplant recipients. Transplantation 1993; 56(1): 75-82.

15. Sonnino RE. RS-61443 prevents graft-versus-host disease but not rejection in allogeneic rat small bowel transplants. Transplant Proc 1992; 24(3): 1190.

16. Shaffer D, Blakely ML, Gottschalk R, Monaco AP. Small bowel transplantation in rats using RS-61443: Effect on GVHD and rejection. Transplant Proc 1992; 24(3): 1159-60.

17. D'Alessandro AM, Rankin M, McVey J, Hafez GR, Sollinger HW, Kalayoglu M, Belzer FO. Prolongation of canine intestinal allograft survival with RS-61443, Cyclosporine, and Prednisone. Transplantation 1993; 55(4): 695-701.

18. Nakajima K, Ochiai T, Nagata M, Suzuki T, Gunji Y, Asano T, *et al.* Effects of triple therapy of Cyclosporine, FK506, and RS-61443 on allogeneic small bowel transplantation in dogs. Transplant Proc 1993; 25(1): 595-6.

19. Canadian Council on Animal Care. Guide to the care and use of Experimental Animals. The Council, 1980.

20. Yatscoff RW, Keenan R, LeGatt DF. Single-dose pharmacokinetics of the new immunosuppressant RS-61443 in the rabbit. Ther Drug Monit 1993; 15(5): 400-4.

21. Association of Official Analytical Chemist (1986): Methods of analysis (23 Ed.). AOAC, Washington, D.C.

22. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1956; 226: 497-509.

23. Elia M, Behrens R, Northrop C, Wraight P, Neale G. Evalation of mannitol, lactulose and ^{51}Cr-labelled ethylenediaminetetra-acetate as markers of intestinal permeability in man. Clin Sci 1987; 73: 197-204.

24. Hamilton I, Rothwell J, Archer D, Axon ATR. Permeability of the rat small intestine to carbohydrate probe molecules. Clin Sci 1987; 73: 189-96.

25. Joyce Loebl. Image analysis: Principles and practice. Great Britain: Joyce Loebl, 1985.

26. Erickson RA, Rivera N. Effect of difluoromethylornithine (DFMO) on NSAID-induced intestinal injury in rats. Dig Dis Sci 1992; 37(12): 1833-9.

27. Pahl MV, Erickson RA, Vaziri ND, Khamiseh G, Oveisi F, Jan K, *et al.* Intestinal morphometry and bile acid-induced mucosal injury in chronic experimental renal failure. J Lab Clin Med 1990; 115: 572-8.

28. Field M, Fromm D, McColl I. Ion transport in rabbit ileal mucosa. I, Na and Cl fluxes and short-circuit current. Am J Physiol 1971; 220(5): 1388-96.

29. Fedorak RN, Chang EB, Madara JL, Field M. Intestinal adaptation to diabetes: Altered Na-dependent nutrient absorption in streptozocin-treated chronically diabetic rats. J Clin Invest 1987; 79: 1571-8.

30. Clarke RM. Mucosal architecture and epthelial cell production rate in the small intestine of th albino rat. J Anat 1970; 107: 519-29.

31. Fedorak RN, Cheeseman CI, Thomson ABR, Porter V. Altered glucose carrier expression: mechanism of intestinal adaptation during streptozocin-induced diabetes in rats. Am J Physiol 1991; 261(4): G585-G591.

CHAPTER VIII

CONCLUSIONS:

THE EFFECTS OF NOVEL IMMUNOSUPPRESSIVE AGENTS ON GROWTH, NUTRITION AND SMALL BOWEL FUNCTION IN NORMAL RATS: ANALYSIS OF THEIR ROLES IN SMALL BOWEL TRANSPLANTATION

Considering the strong immunogenicity of the small bowel and the relatively poor outcomes of clinical small bowel transplantation (SBT) to date, it is obvious that new immunosuppression protocols need to be established. However, the use of more powerful agents can be associated with increased toxicity to the host and the already compromised transplanted organ. It is vital, therefore, to closely study the effects of these agents in order to utilize them clinically in a rational, scientific manner.

Our initial goal at the onset of these studies was to examine the effects of cyclosporin (CsA) and newer immunosuppressive agents on growth, nutrition and small bowel function in rats. The preceding papers have looked at these factors in detail as well as discussed possible mechanisms by which these effects may occur. These will be reviewed here, drawing comparisons and contrasts between the various agents studied. As yet unanswered questions will be posed and possible further investigations required in order to address them, will be proposed. Finally, the potential roles of these agents in immunosuppression for SBT will be discussed.

EFFECTS OF IMMUNOSUPPRESSION ON GROWTH AND NUTRIENT ABSORPTION

As was clearly demonstrated in chapters III through VII, effects long-term administration of the various agents, at the doses used, have variable effects on overall growth in normal rats. CsA and RS61443 resulted in no significant changes, whereas the other agents impaired weight gain.

Deoxyspergualin (DSG) resulted in a moderate impairment, by 26% relative to controls, while FK506 and rapamycin (RAPA) had more profound effects, impairing growth by 79% and 73%, respectively (Table VIII-1, Figure VIII-1).

Growth depends on a complex assimilation and utilization of nutrients, energy, vitamins and minerals, over and above requirements for homeostasis and maintenance of ongoing physiological processes and body cell mass (1). Simplified, we can consider energy only, with the energy available for growth being equivalent to the intake and absorption of nutrient energy less the sum of normal basal metabolic energy requirements and additional metabolic and physiologic energy demands on the organism. Thus, alterations in weight gain can be effected by changes in any one or more of these parameters. Our studies have looked at various aspects of these functions, focusing primarily on nutrient intake and absorption, both *in vivo* and *in vitro* at the functional intestinal and enterocyte levels. Where then, in this equation, do these agents have their effects?

The studies outlined in chapter III demonstrated that despite inducing significant decreases in energy and fat absorption, by 4% and 10%, respectively, CsA had no obvious adverse effects on weight gain compared to controls (Table VIII-1, Figure VIII-1). If we assume that the energy available for growth is the net difference between the total energy consumed and absorbed, and the energy required for basal metabolic requirements, then, by comparing the results of the test animals to the normal controls, we can deduce the potential impact of altered nutrient absorption patterns on growth (2). From this, we can also calculate the amount of energy required to maintain a normal rate of weight gain (expressed as energy required for a gram of weight gain). For clarity, these calculations are outlined in Appendix II of this thesis.

The results of these calculations indicate that the CsA-treated animals were able to compensate for decreased total energy absorption by increasing their feed intake, such that the daily energy available for growth was actually 8.7% greater than that of controls. However, upon calculating the weight gain expected, based on energy available for growth, these animals only gained weight at a rate of 86% of

expected. Thus, they may have also been experiencing additional metabolic and physiologic demands to account for this discrepancy. Where this excess energy was utilized can only be speculated on. It may have been used to promote the observed villus hypertrophy/hyperplasia of the bowel mucosa. Alternatively, as will be discussed in greater detail below, active uptake of nutrients (3OMeG) in the ileum of these rats was relatively inefficient; the enterocytes may have needed to "burn more calories" than normal in order to maintain a normal net substrate uptake.

The negative impact of CsA on fat absorption, as an isolated component of total nutrient energy available for growth, was obvious. Indeed, despite an increase in feed intake, daily energy available from fat, alone, was still less (by 3.4%) than control values and suggest that the decrease in total energy available is primarily the result of this fat malabsorption. It is important to note that these animals were on a relatively low-fat diet (4.7% of the weight of dried feed). With a more physiologic diet such as 20-40% fat composition, the average fat content of North American diets in man (3), the impact of fat malabsorption on decreased energy available for growth would be obviously amplified.

As discussed in chapter III, the effects of CsA on fat malabsorption have been well described and appear to be related to its negative effects on bile secretion (4,5). This affects fat emulsification and subsequent absorption, and may also affect the absorption of fat-soluble vitamins. Specific effects on net *in vivo* protein and carbohydrate absorption were not examined in these studies, although other investigators have found that CsA can produce a reduction in neutral amino acid absorption *in vivo* (6). Future studies need to look at CsA's selective effects on absorption of each of these components of nutrition.

The FK506-treated animals demonstrated profound alterations in growth and nutrient absorption (Table VIII-1, Figure VIII-1). From our calculations in appendix II, it would appear that the energy available for growth was reduced by 16.5% relative to controls. This can be only partially accounted for by a 11% decrease in calories available from fat. As will be discussed below, FK506 induces profound alterations on small intestinal integrity, active uptake of nutrients, and enterocyte energy metabolism--

processes which could thereby negatively alter fat, carbohydrate and protein absorption. As proposed with CsA, specific *in vivo* studies of protein and carbohydrate absorption need to be performed.

Unlike CsA, the mechanism of FK506-induced fat malabsorption has not been entirely elucidated. As discussed in chapter IV, a cholestatic effect of FK506, similar to that induced by CsA, has been debated. *In vitro* studies argue against the clinical significance of this (7). However, *in vivo*, choleritic effects of FK506 in dogs resembles that of CsA (8,9). These observations combined with FK506's similar molecular mechanism of action and similar toxicity profile to CsA suggest that, like CsA, it may have a cholestatic role to play in the fat malabsorption observed. *In vivo* studies of bile salt production and secretion in rats, similar to those already done with CsA, need to be performed.

Weight gain was impaired by 79% in these animals. As discussed above, energy available for growth is equivalent to the intake and absorption of nutrient energy less the sum of normal basal metabolic energy requirements and any additional metabolic and physiologic energy demands on the organism. As total energy available for growth was reduced by only 16.5% in these animals, it would appear that additional energy demands were being imposed. Indeed, these animals gained only 25% of what would be expected, based on their caloric energy available for growth, further indicating that they experienced additional energy demands. Significant hyperreactivity was observed in these animals, with an obvious increase in caloric expenditure. As will be discussed below, absorption of nutrients by the small bowel may be inefficient, resulting in increased work and subsequent energy utilization, by the enterocyte. It is therefore possible that other inefficient and energy-costly metabolic processes my be induced by long-term administration of FK506. Furthermore, at the cellular level, conversion of substrates into energy was shown to be adversely affected by FK506. It would therefore appear that there may be several mechanisms by which this agent imposes excess energy demands and/or inefficient energy utilization in these animals.

RAPA also severely impaired weight gain in the test animals (Table VIII-1, Figure VIII-1). Energy available for growth was decreased by 19% relative to controls. Unlike the FK506-treated rats, this was

not primarily the result of decreased absorption of nutrients, but largely due to a small but significant 6% decrease in feed intake. Although not reflected in the total energy, fat absorption was decreased by 5%. This has not been previously reported as an effect of RAPA, but in view of its adverse effects on function of the ileum, it may be the result of altered enterohepatic circulation of bile salts as theorized in chapter V.

Similar to FK506, the severe 73% impairment in rate of weight gain in the RAPA-treated rats cannot be entirely explained by this reduction in energy available for growth. This actual rate of weight gain was only 33% of expected, based on the calculated energy available for growth, indicating that additional metabolic and/or physiologic demands were experienced by these animals. This will be discussed in greater detail below, but may be related to RAPA's negative effects on intracellular protein synthesis.

Deoxyspergualin induced a moderate 26% impairment of weight gain relative to the control animals (Table VIII-1, Figure VIII-1). Despite a 12% decrease in fat absorption, the calculated total energy absorption, and therefore, energy available for growth, was only minimally affected. Fat malabsorption has not been previously reported with this agent and, as discussed in chapter VI, its mechanism can only be speculated on. As with the three agents discussed above, this may be the result of processes specific to fat absorption. Moderate alterations in liver function have been reported previously with DSG, although significant hepatotoxicity has not been associated with this agent (10). Alternatively, a moderate increase in permeability, and therefore some loss of intestinal integrity, of the ileum was observed in our studies, which could potentially interrupt the enterohepatic circulation of bile salts. Finally, the negative effect on energy production within enterocytes (discussed in more detail, below) may also occur at the biliary canalicular level, inhibiting bile salt secretion, which is an active, energy-dependent process. Further studies to look at these various components of fat absorption need to be performed.

Since poor weight gain in the DSG-treated animals was not due to a decrease in energy available for growth, like the FK506 and RAPA-treated group, additional metabolic and/or physiologic demands may be involved. Indeed, they only gained 75% of the weight expected, based on calculations from the energy available for growth. The significant hyperreactive behavior was likely associated with an increased

caloric energy expenditure. The proportional degree to which this contributed to the excess energy utilization, however, would be difficult to determine in this animal model. In addition, as seen with FK506, conversion of substrates into energy at the enterocyte level was significantly impaired by treatment with DSG. Inefficient energy metabolism on a more global scale may also have resulted in significant "wastage" of energy available for growth.

RS61443 was the most benign agent studied with respect to growth and nutrient absorption, resulting in only a minimal increase in energy absorption relative to controls (Table VIII-1, Figure VIII-1). Upon calculating the energy available for growth, this was increased, by 11%; the change in weight gain, however, was not statistically significant. Nonetheless, this actual weight gain was only 78% of what was expected, as calculate by the energy available for growth, suggesting the presence of additional metabolic or physiological energy demands.

As these animals appeared very healthy and normally active throughout the study, and since RS61443 has a relatively low toxicity profile in rats, it is difficult to determine where this energy was utilized. Perhaps, as a result of RS61443's mechanism of action in blocking purine synthesis in lymphocytes, the necessity of all non-lymphoid cells to utilize a salvage pathway for purine synthesis may have cost excess energy on a global cellular scale. *In vitro* studies of the energy costs of these two pathways to purine synthesis, both with and without the presence of RS61443, would be fascinating.

EFFECTS OF IMMUNOSUPPRESSION ON SMALL BOWEL FUNCTION

Maintenance of the structural and functional integrity of the small intestine is vital to the support of adequate nutrient digestion and absorption, and subsequent growth of the animal. These processes can become altered at various levels. Mucosal surface area for absorption depends on maintenance of mucosal structure, primarily at the villus level. The integrity of the intestine can also be challenged by increases in permeability or "leakiness" of the intercellular tight junctions between enterocytes, allowing already absorbed ions and solutes to diffuse back into the intestinal lumen. Many aspects of nutrient absorption depend on active uptake by the enterocyte. Hence, any adverse effects of these immunosuppressive agents

on these specific energy-dependent processes, or on the physiological "well-being" of the enterocyte may impair these processes. We have seen in the preceding five chapters that various *in vivo* and *in vitro* parameters become altered with long term administration of these immunosuppressive agents. How they affect the overall nutritional processes of the small intestine needs to be understood in order to anticipate potential effects on organ function if used in SBT.

Cyclosporin

CsA, perhaps, has been the most intensely studied of these agents, by both our laboratory and others. As was described in chapter III, villus hypertrophy developed throughout the small intestine in these animals, with no significant alterations in villus density, indicating an increase in overall mucosal surface area (Table VIII-4, Plates 1 and 2). Similar effects on villus size and other components of the bowel wall have been described by others as a result of CsA administration (11,12). What is the mechanism by which these structural alterations occur and how do they correspond to the observed decreases in *in vivo* energy and fat absorption?

These changes may represent an adaptive response of the bowel to enhance nutrient uptake and therefore energy absorption, in response to decreased energy (and fat) absorption and/or increased metabolic demands. The signals for these possible adaptive processes have been discussed in chapter II. They may be hormonal, thereby affecting the bowel as a whole, as reflected in the villus hypertrophy seen throughout the intestine. Alternatively, the presence of increased concentrations of unabsorbed nutrients at the brush-border membrane may have induced this response. The possibility of this is somewhat conflicting when we consider each region of the bowel separately, however, and are applicable primarily to the distal bowel in this model. In the ileum the *in vitro* results revealed significant increased back diffusion of 3OMeG from the serosal to mucosal sides of the bowel (Table VIII-5B, Figure VIII-3B). This appeared to stimulate an adaptive response of the mucosa and enterocyte, possibly to the presence of increased levels of unabsorbed glucose, to upregulate active uptake of substrate in a mucosal-to-serosal direction in order to maintain a normal net uptake (13). Indeed, in the ileum, where adaptive responses

are typically more pronounced (14,15), we did observe a significant increase in active glucose uptake; the morphologic changes in this region of the bowel may therefore reflect an adaptive process to overall decreased nutrient absorption. Phlorizin-binding studies or use of antibodies specific to the glucose transporter may specifically detect alterations in numbers of glucose transporters, in order to correlate them with changes in morphology (16).

In addition, fat malabsorption, with the increased presence of unabsorbed lipid in the bowel, may have had a similar effect in these studies. Indeed, increased levels of fat in the diet have been found by Thomson *et al* to influence intestinal uptake of passively and actively transported solutes, such as glucose, with a corresponding increase in villus height in the ileum only (17).

As discussed earlier, it is important to note that the animals in these studies were fed a low fat (4.7%) diet. Thus, the proportional contribution of fat malabsorption to the 'adaptive' responses observed, may be minimal. It would be important to study these effects using a more physiological diet, with fat concentrations in the range of 20% to 40% of total caloric intake, where one would subsequently expect an augmentation of these adaptive effects. This would make the applicability of these observations to clinical situations more valid, as this would simulate the fat concentrations of a typical North American diet (3).

Alternatively, but less likely, this may be a purely morphological effect, rather than physiological adaptive response to the effects of CsA. This is would not be entirely surprising as it has become well established that morphologic and functional changes of the intestinal mucosa can occur independently (14). The increased villus size may be a reflection of CsA-induced fibroplasia in the connective tissue components of the villus, similar to that seen in CsA-induced gingival hyperplasia (18).

At the enterocyte level, the observed decrease in overall energy absorption may be the result of inhibition of CsA on the Na^+, K^+-ATPase pump of the enterocyte basolateral membrane. Because of CsA's marked hydrophobicity, it is felt to be able to alter the phospholipid content and fluidity of membranes, and may thereby secondarily alter the activity of membrane-bound proteins such as the

Na^+,K^+-ATPase or the sodium-glucose co-transporter (11,19). Indeed, similar effects have been described on the biliary canalicular membrane and renal tubular cells (4,5,20).

Other observed physiological changes induced by CsA in the ileum are interesting. The *in vitro* integrity of the mucosa, as a barrier to back diffusion of already absorbed solutes, appears to be compromised, as reflected in the significant two-fold increase in electrical conductance (Table VIII-5B). However, the *in vivo* results are more difficult to interpret (Table VIII-3, Figure VIII-2). As discussed in chapter III, the CsA treated animals demonstrated a decrease in recovery of both lactulose and mannitol probes relative to controls, suggesting a decrease in intestinal permeability. However, instead, this may reflect an increase in gut motility, as suggested by the decrease in mannitol absorption. Indeed, the lactulose/mannitol ratio in the test animals was comparable to controls, indicating no change in permeability and contrasting the *in vitro* increase in conductance.

Conflicting results with *in vivo* intestinal permeability studies are not uncommon. CsA-induced increases in *in vivo* intestinal permeability have been reported previously, and, in fact, is a mechanism by which transplant rejection can be monitored for (21,22). Sigalet *et al* demonstrated increased urinary recovery of orally administered probes of varying sizes in normal rats treated with CsA. However, the changes were not consistent between differing strains of animals, and the ratios of recoveries of larger probes to smaller molecules (such as lactulose/mannitol) were also difficult to interpret (21).

In order to resolve these inconsistencies, further *in vitro* studies need be done, looking at permeability of these individual probes in Ussing chambers. Such studies would have the advantage of avoiding apparent variations in permeability due to *in vivo* variations in intestinal transit, mucosal water flux, blood and lymphatic flow, renal function and excretion and tissue or luminal metabolism of the probes (23).

Where, then, does CsA exert its actions in altering growth and the nutritional function of the small intestine. It would be naive to assume that only one mechanism is occurring to account for all of the observed changes. As already discussed above, varying effects on morphometry, permeability and nutrient uptake are observed with this agent, all with potentially different mechanisms. These effects may result in

adaptive processes which could incur large energy costs and may account for some of the excess physiological energy demands calculated to have been experienced by these animals. Other interactions between CsA and intestinal function are possible. CsA has been shown to reduce the vascularity of the intestinal microcirculation, similar to its arteriolar vasoconstriction effects in the kidney (24-26). The resultant hypoxia of the enterocytes may adversely affect their cellular processes as well as maintenance of intestinal permeability by maintaining the functional integrity of the intercellular tight junctions. More recently, CsA has been found to inhibit respiration and ATP transport in renal mitochondria as a component of its nephrotoxic effects. Unlike its actions on the Na^+, K^+-ATPase pump, which are hypothesized to be a result of its ability to intercalate within the phospholipid bilayer of the intestinal basolateral membrane, this effect on mitochondrial functions is felt to occur via its intracellular binding protein, cyclophilin. It has been proposed that cyclophilin maintains a nonspecific inner membrane permeability pore of the mitochondria in an open configuration (28). Binding of CsA to cyclophilin prevents this interaction and may inhibit ATP transport, Ca^{2+}/Pi-induced mitochondrial swelling and Ca^{2+} release, which may then contribute to the drug's nephrotoxicity (25). Similar *in vitro* studies of enterocytes may indicate whether these processes occur in and may be potentially toxic to small intestine.

FK506

We have seen from the results of our studies with FK506 that, in addition to significant toxic effects not related to the gastrointestinal system, this agent, at the dose utilized, can induce significant alterations in small intestinal integrity and function. As discussed above, it is likely to also have more global effects, as the 16.5% decrease in calculated energy available for growth, cannot fully account for the profoundly impaired weight gain of the treated animals.

Indeed, a loss of integrity of the small intestine was clearly shown in our *in vitro* studies by the marked increase in electrical conductance and "backwards" serosal-to-mucosal flux of 3OMeG, throughout the bowel, most pronounced in the ileum (Tables VIII-5A and 5B, Figures VIII-3A and 3B). Again, as with the CsA studies, the *in vivo* intestinal permeability studies produce a more mixed picture

(Table VIII-3, Figure VIII-2). Corresponding with an increase in permeability, two-fold increases in ^{99}Tc-DTPA, lactulose and mannitol recoveries were observed. However, it is possible that these increases may reflect a decrease in gut motility, allowing longer exposure time of the probes to the transcellular pores and intracellular tight junctions, rather than an absolute increase in intestinal permeability. Indeed, the lack of an increase in the lactulose/mannitol ratio would suggest this. Unfortunately, as explained previously, *in vivo* permeability studies may be fraught with confounding variables of variations in intestinal transit, mucosal water flux, blood and lymphatic flow, renal function and excretion and tissue or luminal metabolism of the probes (23). *In vitro* studies to measure the passage of these probes, directly, need to be performed.

As described in chapter IV, increased permeability may account for the profound back-flux of 3OMeG. In turn, this may stimulated an adaptive process of the mucosa, with an upregulation of active glucose transport and/or increase paracellular movement of glucose to the serosal side (13), resulting in the proportionally enhanced forward flux observed. Again, corresponding to the effects on electrical conductance, these changes were most pronounced in the ileum. Although our *in vitro* studies suggest that the full adaptation of active uptake may have occurred in the jejunum, in that net 3OMeG uptake was at least maintained, the *in vivo* results of overall decreased energy absorption suggest the contrary. Significant to these effects is the large energy expense to the bowel that this "cycling" of substrate between the mucosal and serosal surfaces may have cost. This could potentially have other negative effects on nutrient digestion, absorption or processing, not otherwise directly investigated in these studies.

The most significant finding from these studies, however, is the profound decrease in energy production by the enterocyte mitochondria in the FK506-treated animals (Table VIII-6, Figure VIII-4). Similar alterations of mitochondrial energy metabolism have been reported by others in hepatocytes and renal cells (28,29). The stage at which this occurs, in the process of substrate utilization and subsequent ATP production, can only be speculated on. Our results indicate that the decrease in both glucose and pyruvate substrate utilization, as measured by CO_2 production, is approximately proportional to the

decrease in ATP production. Thus, it would appear the FK506 exerts its effects early in this process, at or between steps involved in uptake of substrate into the mitochondria and subsequent CO_2 production via the tricarboxylic acid (TCA) cycle (30). More detailed studies, perhaps looking at the rates of production of various components of these reactions, such as acetyl-CoA and the numerous constituents of the TCA cycle, may further delineate FK506's site of action.

This adverse effect of FK506 on energy production is fascinating. As described above, alterations of mitochondrial energy production are also seen with CsA. However, the molecular mechanisms by which this occurs is likely different. Like CsA, FK506 inhibits respiration and ATP net transport in renal mitochondria (27). However, this is not related to any alterations in the cyclophilin-dependent open state of the nonspecific inner membrane permeability pore of the mitochondria as are induced by CsA. Indeed, unlike CsA, FK506 has no effects on Ca^{2+}/Pi^{-}-induced swelling of renal mitochondria (27).

Decreased energy availability to the enterocyte was not evident functionally in the *in vitro* 3OMeG-flux studies performed. In fact, as discussed above, markedly elevated energy utilization by the enterocytes may be ongoing in an attempt to counteract loss of net substrate uptake secondary to increased permeability. Decreased energy, however, may explain the increase in intestinal permeability. Mandel *et al* have shown that the functional integrity of intraepithelial tight junctions is strongly dependent on energy (31). Interestingly, Bjarnason *et al* have demonstrated similar effects with the nonsteroidal anti-inflammatory drug (NSAID), indomethecin. In human subjects, urinary excretion of [^{51}Cr]EDTA is increased, indicating increased intestinal permeablity, *in vivo* (32). At the cellular level, this is associated with an uncoupling of oxidative phosphorylation within the enyterocytes (33,34). Similar to the results found in our studies, the authors also postulate that the increased permeability effects are secondary to the resultant loss of intercellular tight junction integrity, which is an energy-dependent process (35). Specific studies such as those outlined in this thesis, to see if these changes would lead to similar alterations in glucose fluxes as seen with FK506 have not been published, but would be very interesting.

Energy is also required for most other cellular processes, including protein synthesis. Thus, any energy deficit may negatively affect these processes and inhibit the overall maintenance of body cell mass. This may be especially so with cells characterized by rapid turnover, such as the hematopoietic system, as reflected in the anemia and leukopenia observed in the treated animals (Table VIII-2).

These effects of FK506 on energy metabolism need to be further studied. It is likely that they occur globally as opposed to being isolated to the enterocyte as studied here. Similar studies using cell cultures of various cell populations need to be done. Whether this is an acute effect of FK506 or occurs only after chronic administration needs to be studied in order to determine if this agent can be safely used for induction and/or maintenance immunosuppressive therapy. Finally, if FK506 does act globally, its effects on the energy metabolism of the organism as a whole needs to be studied. This could be done through the determination of the respiratory quotient in FK506-treated animals, as a measure of oxidative energy production (3).

FK506 is indeed an interesting agent. With a similar proposed mechanism of action as CsA, it has also been found to have a similar toxicity profile. However, at the molecular level of these adverse effects, these two agents appear to have marked differences. Immunologically, they bind to different intracellular proteins, yet exert the same effect on IL-2 production. Both affect mitochondrial energy production, although likely acting at different sites (27). Both inhibit ATP-dependent bile-salt transport in hepatocyte canalicular membranes, but unlike CsA, FK506 requires concentrations to do so which are 1000-fold higher than recommended clinical serum levels (7). Unlike CsA, which is extremely hydrophobic and potentially able to act by altering the fluidity and phospholipid content of membranes and membrane-bound proteins, FK506 lacks these biochemical properties (36).

Rapamycin

As discussed earlier, RAPA's adverse effects on weight gain cannot be explained by a decrease in nutrient absorption; the primary cause of the 19% reduction in energy available for growth appears to be related to a reduction in feed intake. The effects of this agent on the small intestine correlate well with

this, being small and isolated to only the ileum. As shown in chapter V, RAPA induces significant alterations in villus morphology resulting in a decrease in mucosal surface area in this region of the bowel (Table VIII-4, Plate 2). Functionally, this correlates with an obliteration in net uptake of 3OMeG (Table VIII-5B, Figure VIII-3B) and may be related to impaired enterohepatic circulation of bile salt resulting in moderate fat malabsorption.

Unlike CsA and FK506, which may directly affect enterocyte function by alterations in active nutrient uptake processes or enterocyte energy metabolism, RAPA's adverse effects may be directly related to its immunological mechanism of action. As discussed in chapter II, RAPA is felt to impair activation of protein kinases involved in the late G1/S phase transition of IL-2 activated at-cells. It has also been found to block these signal-transduction systems in non-lymphoid cells (37,38). Similarly, it may adversely affect the morphological and subsequent functional aspects of the small intestine. Indeed, Francavilla *et al* demonstrated that RAPA significantly inhibits small intestinal regeneration after a 40% resection, excluding the jejunum, as described in chapter V (39). *In vitro* studies looking at RAPA's effects on protein synthesis and growth of enterocytes, in cell culture, may show this more clearly.

Our studies indicated a ileum-specific effect, however, further studies, perhaps looking at RAPA's effects on regeneration of segmental resections from varying regions of the bowel, may be needed to substantiate this. In addition, although the development of anemia was not obvious in these animals, future studies should investigate vitamin B12 absorption, a function also specific to the ileum.

Deoxyspergualin

Like Rapamycin, DSG negative effects on weight gain were unlikely to be primarily a result of alterations in small intestinal function. As discussed above, despite a 12% decrease in fat absorption, the calculated total energy absorption, and therefore, energy available for growth, was only minimally affected in these animals This corresponds well to the minimal and nonspecific effects observed in both the *in vivo* and *in vitro* studies of intestinal function. Except for moderate increases in permeability to ^{99m}Tc-DTPA and electrical conductance of the ileum (the former only barely reaching statistical significance)

(Table VIII-3, Figure VIII-2), all other study results on the small bowel itself were small and insignificant. The 75% impairment of weight gain relative to what would be expected, based on calculated energy available for growth, however, indicates other metabolic and/or physiological energy demands are being imposed on these animals.

DSG's effects on energy production by the enterocyte were somewhat surprising, but may explain this poor growth observed in the test animals (Table VIII-6, Figure VIII-4). As discussed earlier, energy available for growth is equivalent to the intake and absorption of nutrient energy less the sum of normal basal metabolic energy requirements and additional metabolic and physiologic energy demands on the organism. Our studies indicate that conversion of substrates into energy at the enterocyte level is significantly impaired by treatment with DSG. This inefficient energy metabolism may result in significant "wastage" of potential energy available for growth, especially if occurring on a more global scale. That is, although energy, in the form of calories, was absorbed and therefore calculated to be available for growth, they may have been inefficiently utilized in conversion into true energy, in the form of ATP. Interestingly, this did not have any effect on enterocyte function with respect to nutrient absorption (Tables VIII-5A and 5B, Figures VIII-3A and 3B). As with FK506, however, it may have impaired the functional integrity of intracellular tight junctions resulting in an increase in permeability and electrical conductance (31), although these effects were small and appeared limited to the ileum. Also, some indication of toxicity may be reflected in the small but significant decreases in villus size seen throughout the intestine in these animal (Table VIII-4). It is additionally possible that some functional impairment of active nutrient uptake by the enterocyte may have occurred, but, by the time of *in vitro* study, had been adapted for. [i.e. Perhaps, this may have involved an upregulation of numbers of glucose transporters, albeit all with relatively less efficiency than normal. Or, there may have been an adaptive recruitment of new but different glucose transporters which have higher affinities for substrate or are able to move glucose across the enterocyte at an increased rate or are less dependent on energy (13).] Studies to look at enterocyte nutrient absorptive function after, perhaps after one week of DSG administration

(before any adaptive response can develop), may be beneficial to further clarify this. Indeed, this would be important to assess, since clinically, DSG is likely to be used on a short-term basis for either immunosuppression induction or as rescue therapy for rejection. Perhaps, like its hematopoietic toxicity (Table VIII-2), these effects may be reversible and therefore not as much a concern if it is to be used in SBT (40).

RS61443

As discussed above, RS61443 was the most benign agent studied with respect to growth and nutrient absorption. Its effects on the small intestine, likewise, were minimal. Indeed, the only effects seen in these studies was a small but significant inhibition of active 3OMeG uptake by the jejunum, which appeared to be compensated for by the ileum (Tables VIII-5A and 5B, Figures VIII-3A and 3B). Correspondingly, *in vivo* energy absorption in these animals was comparable to controls (Table VIII-1). It was interesting that this was associated with an increase in villus size in the jejunum (Table VIII-4, Plate 1). It may be that these morphological changes were purely structural and not associated with any corresponding increase in glucose transporters per villus. Subsequently, the decrease in villus density may have resulted in a net reduction of transporters available. Studies to asses this directly, such as [^{3}H]-Phlorizin-binding may be helpful (16).

As RS61443's mechanism of action is lymphocyte-specific, and other somatic cells, including enterocytes, have a salvage pathway for synthesis of guanosine monophosphate (41,42), this relative lack of adverse effects directed towards the small bowel is understandable. The jejunum-specific effects observed are, as yet, beyond explanation based on the studies performed and this agents known mechanism of action.

THE ROLES OF NOVEL IMMUNOSUPPRESSIVE AGENTS IN SBT

Small bowel transplantation offers a potential cure for those patients suffering from short bowel syndrome As has been the theme throughout this thesis, the success of SBT is limited by the lack of adequate immunosuppression protocols, and the development of more powerful agents and/or regiments is

required. However, success also depends on adequate functioning of the transplanted bowel in order to maintain the nutritional needs of the recipient. Hence, as well as a relatively low toxicity profile on other organs and functions of the patient, immunosuppression should not adversely affect the integrity and function of the transplanted intestine, which may already be compromised secondary to the transplantation process itself.

CsA has already proven successful in some cases of isolated small bowel and small bowel/liver transplants (43,44). As discussed in chapter II, it is associated with significant nephrotoxicity and neurotoxicity, cholestatic actions, and the development of infections and malignancies secondary to overimmunosuppression, especially when used for transplantation of such a highly immunogenic organ as the bowel. In these studies, the dosage used was likely appropriate, and corresponds well to the parenteral doses utilized in clinical transplantation. Subsequently, its effects directly on the small bowel were relatively small, with minimal adverse effects on overall growth and well-being of the animals. However, rather than being employed alone, CsA will likely be most useful when used in combination with other agents having alternate immunological mechanisms of action, as will be discussed below. In this way, its dosage, and therefore adverse effects, can be minimized, while still obtaining adequate immunosuppression. In this way, it could therefore be considered safe and efficacious to use in SBT.

FK506, at the dosage used, was associated with significant toxicity and poor weight gain, and induced profound alterations on intestinal function with respect to nutrient absorption and enterocyte energy metabolism. However, the dose used is now considered almost ten-fold greater than the suggested dose in humans, and the serum levels obtained were, indeed, four to ten times greater than the recommended levels (36). Thus, before determining whether this agent may have deleterious effects on the bowel when used in SBT, these studies need to be repeated utilizing much lower doses in the range of 0.3 mg/kg/d, as is presently employed in clinical transplantation. FK506 has been used in the largest series of intestinal transplant patients to date and reports of nutritional function in these patients appear favorable (45,46). Its reported efficacy as an immunosuppressant for SBT has also proved better than any other

immunosuppressive regimen studied to date, although careful clinical trials, comparing it to agents such as CsA, need to be performed before such conclusions can truly be derived. In addition as discussed in chapter II and seen in our studies, it still has significant nephrotoxic and neurotoxic effects, and is strongly associated with the development of PTLD, especially when used in pediatric patients (47,48).

RAPA was also associated with a severe impairment of growth, which has been reported by others in various animal models of transplantation. As explained earlier, however, these effects, were not directly attributable to alterations in nutrient absorption or small bowel function, but more likely resulted from alterations in protein synthesis. Although comparable to doses employed by others in the literature when used as a sole agent, with comparable serum levels, the dosage of RAPA utilized in these studies was obviously severely toxic to the animals. Thus, clinically, RAPA would be most effective when used at subtherapeutic doses, as when in combination with CsA. Differing molecular mechanisms of action and toxicity spectrums further support this. Indeed, the synergism between these two agents in a rat SBT model has been demonstrated by Chen *et al*, with a dose as low as 0.2 mg/kg/d, i.v. (49). However, RAPA's effects on protein synthesis, especially the potential relationship to impairment of organ regeneration, as observed with resected small intestine by Francavilla *et al* (39), needs to be studied further. Importantly, a dose-relationship of these effects needs to be established. This should guide further studies. Indeed, RAPA appears to be a powerful agent, especially when used in synergistic combination with agents such as CsA, and should not be discarded. If employed at lower doses, and for briefer time periods, such as only in the induction phase of immunosuppression, it may be a useful agent to be used in SBT.

DSG also impaired weight gain and was associated with moderate toxicity to the animals. However, these latter effects were primarily related to the hematopoietic and system, a well-known adverse effect of this drug. From the studies performed, its effects on small bowel function were minimal, despite impaired energy metabolism by the enterocytes. Interestingly, despite this occurring on the same scale as seen in the FK506 animals, the observed alterations in permeability were not as profound, perhaps indicating the

involvement of another co-mechanism in the FK506 group. Likewise, the effects on growth were equally less. With an alternate immunological mechanism of action than CsA or FK506, primarily acting after the activation stage of T-lymphocytes in the rejection response, and with a different spectrum of toxicity, DSG is a likely candidate to be used in combination with either of these agents. Until the possibility of synergistic adverse effects by both DSG and FK506 on energy metabolism is ruled out, however, the likely partner would be CsA. Indeed, such a combination has been found to be synergistically immunosuppressive in a rat cardiac transplant model by Gannedahl *et al* (50,51) and effective in clinical islet transplantation by Gores *et al* (52), and warrants further investigation for SBT.

Finally, RS61443, with its minimal effects on small bowel function, is also a promising agent to be potentially employed in SBT. With a lymphocyte-specific molecular mechanism of action which is qualitatively different than any of the agents already discussed, perhaps it is not surprising that its effects on animal well-being, growth, nutrient absorption, and small bowel function were minimal. More significantly, the serum drug levels were comparable, if not higher, to those required for successful canine SBT (53) (as outlined in chapter VII), still with relatively little toxicity observed. RS61443's alternate mechanism of action makes it favorable to be used in combination with any of the previous agents discussed, and indeed, it has been demonstrated to act synergistically with CsA in canine liver and kidney, and rat cardiac transplantation (54-56) and complements the action of CsA in canine SBT (53). Furthermore, if used in a manner similar to its cousin, brequinar sodium (BQR) (which, as discussed in chapter II, selectively inhibits pyrimidine synthesis in lymphocytes), it may be effectively synergistic when administered in combination with low dose CsA and RAPA, as demonstrated by Kahan *et al* in rat cardiac transplantation (57).

Table VIII-1

Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin and RS61443 on Weight gain, Feed intake and Energy and Fat absorption

	Weight Gain (g/d)		Feed Intake (g/kg/d)		% Energy Absorption		% Fat Absorption	
Control	3.15 ± 0.07		69.42 ± 1.11		81.3 ± 0.6		76.1 ± 0.9	
CsA	2.95 ± 0.11	6%↓ p = 0.364	74.52 ± 0.84	***7%↑** p < 0.001	78.3 ± 0.6	***4%↓** p < 0.001	68.3 ± 0.9	***10%↓** p < 0.001
FK506	0.65 ± 0.14	***79%↓** p < 0.001	76.00 ± 1.68	***9%↑** p = 0.002	69.5 ± 1.0	***14%↓** p < 0.001	61.0 ± 1.0	***20%↓** p < 0.001
Rapamycin	0.84 ± 0.08	***73%↓** p < 0.001	65.16 ± 0.66	***6%↓** p = 0.002	80.1 ± 1.5	1%↓ p = 0.345	71.9 ± 2.2	***5%↓** p = 0.045
Deoxyspergualin	2.33 ± 0.09	***26%↓** p < 0.001	69.75 ± 1.05	0%↔ p = 0.739	80.3 ± 1.4	1%↓ p = 0.391	66.9 ± 1.8	***12%↓** p < 0.001
RS61443	2.76 ± 0.21	12%↓ p = 0.172	70.90 ± 1.00	2%↑ p = 0.297	83.1 ± 0.5	***2%↑** p = 0.011	76.1 ± 0.6	0%↔ p = 0.965

Values are means ± SEM

* significant difference compared to controls ($p<0.05$).

% values indicated relative to controls (↑, increase; ↓, decrease).

Table VIII-2

Effects of Cyclosporin, FK506 and Deoxyspergualin on Serum and Hematological Values

	Na^+ (mmol/l)	K^+ (mmol/l)	Creatinine (mmol/l)	Glucose (mg/dl)	WBC (10^3c/mm^3)	RBC (10^3c/mm^3)	HgB (g%)	Hct (%)
Control 1	142.7 ± 0.9	9.3 ± 0.9	0.63 ± 0.02	n/a	n/a	n/a	n/a	n/a
Control 2	143.2 ± 0.6	7.6 ± 0.8	0.42 ± 0.04	128.5 ± 0.5	7.8 ± 0.6	8.6 ± 0.2	16.4 ± 0.2	43.8 ± 0.9
Cyclosporin†	136.3 ± 0.5 ***4%↓** p<0.001	17.6 ± 1.2 ***88%↑** p<0.001	0.43 ± 0.14 33%↓ p=0.11	n/a	n/a	n/a	n/a	n/a
FK506‡	138.8 ± 1.7 3%↓ p>0.1	10.1 ± 2.1 32%↑ p>0.1	0.81 ± 0.15 93%↑ p>0.1	113.6 ± 12.8 12%↓ p=0.43	5.3 ± 1.0 ***32%↓** p=0.03	7.5 ± 0.4 ***13%↓** p=0.026	14.9 ± 0.6 *** 9%↓** p=0.01	38.6 ± 1.4 ***12%↓** p=0.006
Deoxyspergualin‡	143.4 ± 0.6 0%↔ p=0.73	6.1 ± 0.7 19%↓ p=0.20	0.61 ± 0.05 45%↑ p=0.04	118.4 ± 17.7 8%↓ p=0.75	4.4 ± 0.8 ***43%↓** p=0.003	5.9 ± 0.3 ***32%↓** p<0.001	12.4 ± 0.4 ***25%↓** p<0.001	31.7 ± 1.4 ***28%↓** p<0.001

Values are means ± SEM

* significant difference compared to controls ($p<0.05$)

† compared to Control 1; ‡ compared to Control 2

% values indicated relative to controls (↑, increase; ↓, decrease).

WBC, white blood cell count; RBC, red blood cell count; HgB, hemaglobin; Hct, hematocrit.

Table VIII-3
Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin, and RS61443 on *In Vivo* Intestinal Permeability

	^{99}Tc-DTPA		Mannitol		Lactulose		Lactulose/Mannitol	
Control 1	n/a		3.77 ± 0.22		1.40 ± 0.11		0.41 ± 0.03	
Control 2	2.24 ± 0.18		1.97 ± 0.11		0.81 ± 0.04		0.42 ± 0.06	
CsA†	n/a		2.84 ± 0.24	***25%↓** p<0.013	1.19 ± 0.07	15%↓ p=0.11	0.43 ± 0.03	4%↑ p=0.668
FK506‡	4.35 ± 0.90	***94%↑** p=0.009	4.49 ± 0.60	***128%↑** p<0.001	1.63 ± 0.27	***100%↑** p<0.001	0.35 ± 0.02	***16%↓** p=0.020
Rapamycin†	n/a		1.94 ± 0.26	***49%↓** p<0.001	0.98 ± 0.12	***30%↓** p=0.027	0.48 ± 0.04	16%↑ p=0.083
Deoxyspergualin‡	3.71 ± 0.66	66%↑ p=0.055	1.98 ± 0.13	1%↑ p=0.957	0.79 ± 0.08	2%↓ p=0.121	0.41 ± 0.04	2%↓ p=0.448
RS61443†	n/a		2.42 ± 0.33	***36%↓** p=0.005	1.03 ± 0.15	26%↓ p=0.086	0.43 ± 0.02	3%↑ p=0.197

Data is presented as percent recovery of orally administered marker in urine; mean ± SEM
* significant difference compared to controls ($p<0.05$); † compared to Control 1; ‡ compared to Control 2
% values indicated relative to controls (↑, increase; ↓, decrease).

Table VIII-4
Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin and RS61443 on Villus Morphometry and Density

	JEJUNUM				ILEUM			
	Width ($\mu m \times 10^2$)	**Height ($\mu m \times 10^2$)**	**Area ($\mu m^2 \times 10^2$)**	**# Villi per 20X field**	**Width ($\mu m \times 10^2$)**	**Height ($\mu m \times 10^2$)**	**Area ($\mu m^2 \times 10^2$)**	**# Villi per 20X field**
Control 1	1.66 ± 0.05	4.74 ± 0.12	4.67 ± 0.19	6.58 ± 0.52	1.70 ± 0.06	3.84 ± 0.14	4.13 ± 0.23	5.26 ± 0.41
Control 2	2.00 ± 0.05	6.86 ± 0.07	8.03 ± 0.21	5.41 ± 0.19	2.06 ± 0.06	4.07 ± 0.07	4.94 ± 0.17	4.65 ± 0.33
CsA†	1.96 ± 0.06 ***18%↑** p<0.001	5.74 ± 0.08 ***21%↑** p<0.001	6.81 ± 0.28 ***46%↑** p=0.001	5.61 ± 0.04 15%↓ p=0.139	1.88 ± 0.06 ***11%↑** p=0.031	4.17 ± 0.06 ***9%↑** p=0.003	4.69 ± 0.18 ***14%↑** p=0.029	5.20 ± 0.23 1%↓ p=0.902
FK506‡	1.90 ± 0.06 5%↓ p=0.25	6.80 ± 0.08 1%↓ p=0.54	7.82 ± 0.24 3%↓ p=0.47	5.63 ± 0.34 4%↑ p=0.573	2.15 ± 0.10 4%↑ p=0.595	1.13 ± 0.07 1%↑ p=0.26	5.24 ± 0.27 6%↑ p=0.851	5.31 ± 0.20 14%↑ p=0.188
Rapamycin†	1.55 ± 0.04 6%↓ p=0.212	5.32 ± 0.10 ***12%↑** p<0.001	4.97 ± 0.16 6%↑ p=0.289	6.65 ± 0.42 1%↑ p=0.928	1.42 ± 0.07 ***16%↓** p<0.001	3.14 ± 0.04 ***18%↓** p=0.007	2.65 ± 0.12 ***36%↓** p<0.001	6.27 ± 0.54 19%↑ p=0.187
Deoxyspergualin‡	1.97 ± 0.06 1%↓ p=0.773	6.64 ± 0.11 3%↓ p=0.094	6.95 ± 0.21 ***13%↓** p=0.001	5.53 ± 0.16 2%↑ p=0.693	1.90 ± 0.08 8%↓ p=0.059	3.93 ± 0.07 3%↓ p=0.269	4.22 ± 0.17 ***16%↓** p=0.007	5.54 ± 0.26 19%↑ p=0.106
RS61443†	1.82 ± 0.05 ***9%↑** p=0.004	6.16 ± 0.06 ***30%↑** p<0.001	6.56 ± 0.17 ***40%↑** p<0.001	4.46 ± 0.25 ***32%↓** p=0.003	1.70 ± 0.07 0%↔ p=0.954	3.74 ± 0.06 3%↓ p=0.676	3.84 ± 0.15 7%↓ p=0.890	4.75 ± 0.49 10%↓ p=0.483

Data is presented as villus width, height from lamina propria and saggital section area; expressed as mean ± SEM

* significant difference compared to controls ($p<0.05$)

% values indicated relative to controls (↑, increase; ↓, decrease); † compared to Control 1; ‡ compared to Control 2

Table VIII-5A

Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin and RS61443 on *In Vitro* 3OMeG fluxes and Electrophysiological Parameters in the Jejunum

	J_{ms}	J_{sm}	J_{net}	PD	I_{sc}	G
Control	1.24 ± 0.08	0.84 ± 0.06	0.41 ± 0.05	1.70 ± 0.12	39.29 ± 3.16	22.56 ± 1.54
CsA	1.51 ± 0.16 22%↑ p=0.093	0.94 ± 0.07 12%↑ p=0.285	0.57 ± 0.12 39%↑ p=0.157	2.07 ± 0.11 21%↑ p=0.119	47.60 ± 3.69 21%↑ p=0.123	24.95 ± 2.11 11%↑ p=0.379
FK506	2.35 ± 0.14 ***90%↑** p<0.001	1.61 ± 0.14 ***92%↑** p<0.001	0.74 ± 0.09 ***80%↑** p<0.001	1.31 ± 0.17 23%↓ p=0.059	32.78 ± 3.91 17%↓ p=0.118	42.73 ± 3.44 ***89%↑** p<0.001
Rapamycin	1.35 ± 0.06 9%↑ p=0.632	0.78 ± 0.17 7%↓ p=0.742	0.57 ± 0.21 39%↑ p=0.291	1.71 ± 0.08 1%↑ p=0.998	64.78 ± 6.63 ***65%↑** p=0.002	5.98 ± 5.56 15%↑ p=0.471
Deoxyspergualin	1.31 ± 0.06 9%↓ p=0.317	0.83 ± 0.10 1%↓ p=0.736	0.29 ± 0.08 29%↓ p=0.147	1.47 ± 0.35 14%↓ p=0.424	32.17 ± 2.32 18%↓ p=0.117	23.73 ± 2.61 5%↑ p=0.68
RS61443	0.93 ± 0.11 ***25%↓** p≤0.050	0.85 ± 0.09 1%↑ p=0.913	0.08 ± 0.08 ***80%↓** p=0.001	1.73 ± 0.37 2%↑ p=0.939	26.70 ± 3.24 ***32%↓** p=0.019	22.28 ± 2.02 1%↓ p=0.930

Values are means ± SEM. J_{ms}, flux from mucosa to serosa; J_{sm}, flux from serosa to mucosa; J_{net}, numerical difference between J_{ms} and J_{sm} in μmol/cm²/hr; PD, potential difference in mV; Isc, short-circuit current in μAmp/cm²/hr; G, conductance in mS/cm².

% values indicated relative to controls (↑, increase; ↓, decrease).

*significant difference compared to controls ($p<0.05$)

Table VIII-5B

Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin and RS61443 on *In Vitro* 3OMeG fluxes and Electrophysiological Parameters in the Ileum

	J_{ms}	J_{sm}	J_{net}	PD	I_{sc}	G
Control	1.19 ± 0.08	0.82 ± 0.08	0.36 ± 0.06	2.26 ± 0.15	40.86 ± 2.59	18.25 ± 1.50
CsA	1.58 ± 0.11 ***33%↑** p=0.048	1.25 ± 0.11 ***52%↑** p=0.006	0.33 ± 0.05 8%↓ p=0.717	0.88 ± 0.16 ***61%↓** p<0.001	29.45 ± 4.33 ***28%↓** p=0.029	36.18 ± 3.31 ***98%↑** p<0.001
FK506	3.14 ± 0.45 ***164%↑** p<0.001	3.10 ± 0.54 ***278%↑** p<0.001	0.04 ± 0.16 89%↓ p=0.194	0.38 ± 0.11 ***83%↓** p=0.003	24.77 ± 4.03 ***39%↓** p<0.001	65.63 ± 10.71 ***260%↑** p<0.001
Rapamycin	1.06 ± 0.13 11%↓ p=0.436	1.07 ± 0.14 30%↑ p=0.125	-0.01 ± 0.07 ***100%↓** p<0.001	2.12 ± 0.34 6%↓ p=0.482	37.56 ± 2.16 8%↓ p=0.378	20.82 ± 1.22 14%↑ p=0.228
Deoxyspergualin	1.33 ± 0.12 12%↑ p=0.403	0.87 ± 0.11 6%↑ p=0.676	0.46 ± 0.11 28%↑ p=0.382	2.70 ± 0.37 19%↑ p=0.625	45.42 ± 6.84 11%↑ p=0.916	24.19 ± 1.80 ***33%↑** p=0.019
RS61443	1.59 ± 0.05 ***34%↑** p=0.018	0.87 ± 0.08 6%↑ p=0.732	0.72 ± 0.10 ***100%↑** p=0.006	2.43 ± 0.01 8%↑ p=0.229	61.74 ± 3.85 ***51%↑** p<0.001	21.95 ± 1.37 20% p=0.171

Values are means ± SEM. J_{ms}, flux from mucosa to serosa; J_{sm}, flux from serosa to mcosa; J_{net}, numerical difference between J_{ms} and J_{sm} in μmol/cm^2/hr; PD, potential difference in mV; Isc, short-circuit current in μAmp/cm^2/hr; G, conductance in mS/cm^2.

% values indicated relative to controls (↑, increase; ↓, decrease).

*significant difference compared to controls ($p<0.05$);

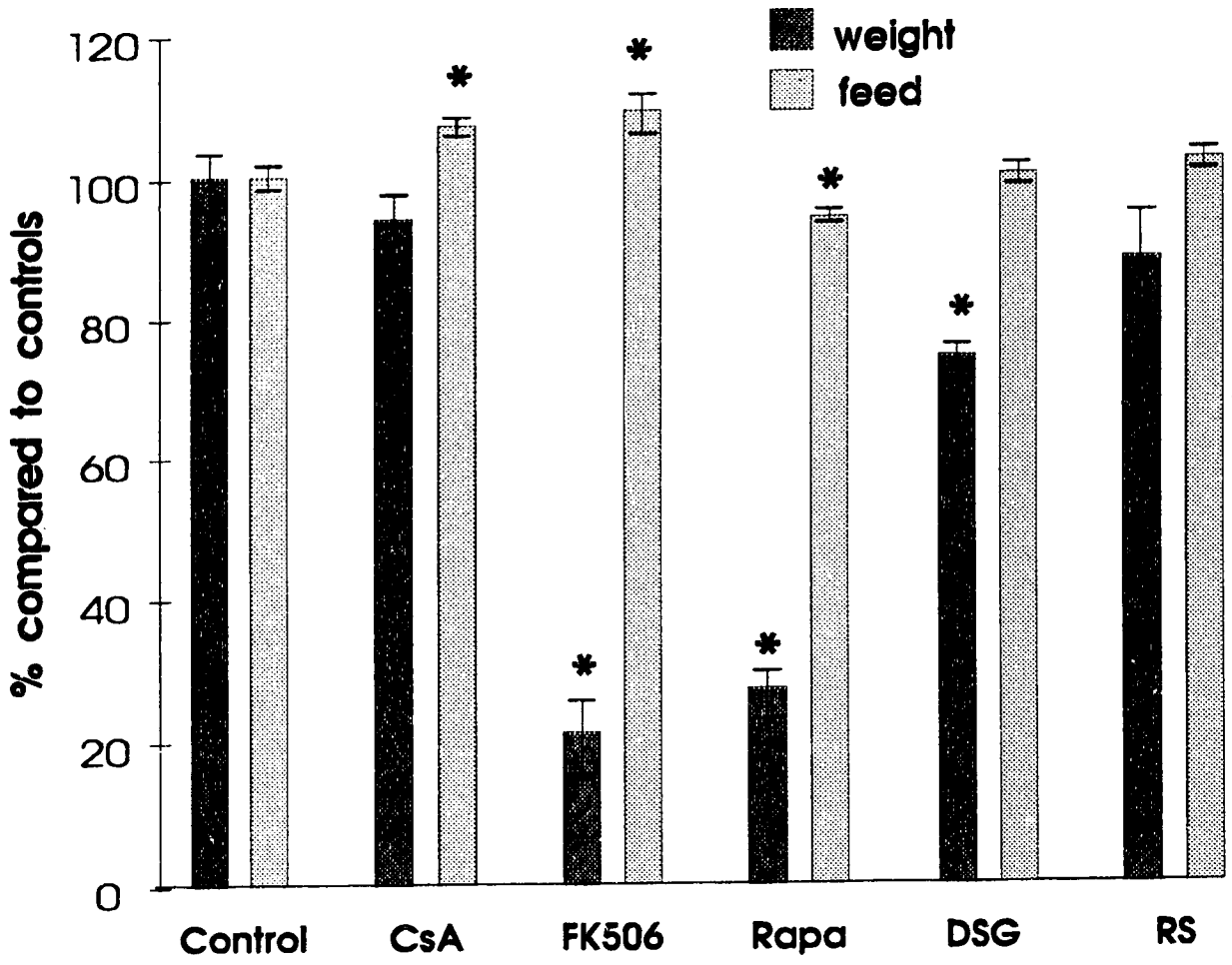
Table VIII-6
Effects of FK506 and Deoxyspergualin on *In Vitro* Mitochondrial Energy Production

	[^{14}C]Glucose utilization ng/hr/mg protein	[^{14}C]Pyruvate utilization nmol/hr/mg	Lactate production mg/hr/mg protein	Pyruvate production mg/hr/mg protein	ATP production nmol/μg protein
Control	24.20 ± 1.21	1.19 ± 0.15	0.10 ± 0.02	0.0067 ± 0.0006	1.32 ± 0.12
FK506	11.11 ± 3.42 ***45%↓** p=0.005	0.60 ± 0.11 ***50%↓** p=0.007	0.10 ± 0.02 0%↔ p=0.89	0.0098 ± 0.0038 12%↑ p=0.50	0.89 ± 0.04 ***33%↓** p=0.015
Deoxyspergualin	9.55 ± 2.13 ***60%↓** p<0.001	0.32 ± 0.14 ***73%↓** p<0.001	0.10 ± 0.01 0%↔ p=0.97	0.0078 ± 0.0011 8%↑ p=0.48	0.80 ± 0.14 ***39%↓** p=0.029

Values are means ± SEM
* significant difference compared to controls ($p<0.05$)
% values indicated relative to controls (↑, increase; ↓, decrease).

Figure VIII-1

Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin and RS61443 on Weight gain and Feed intake

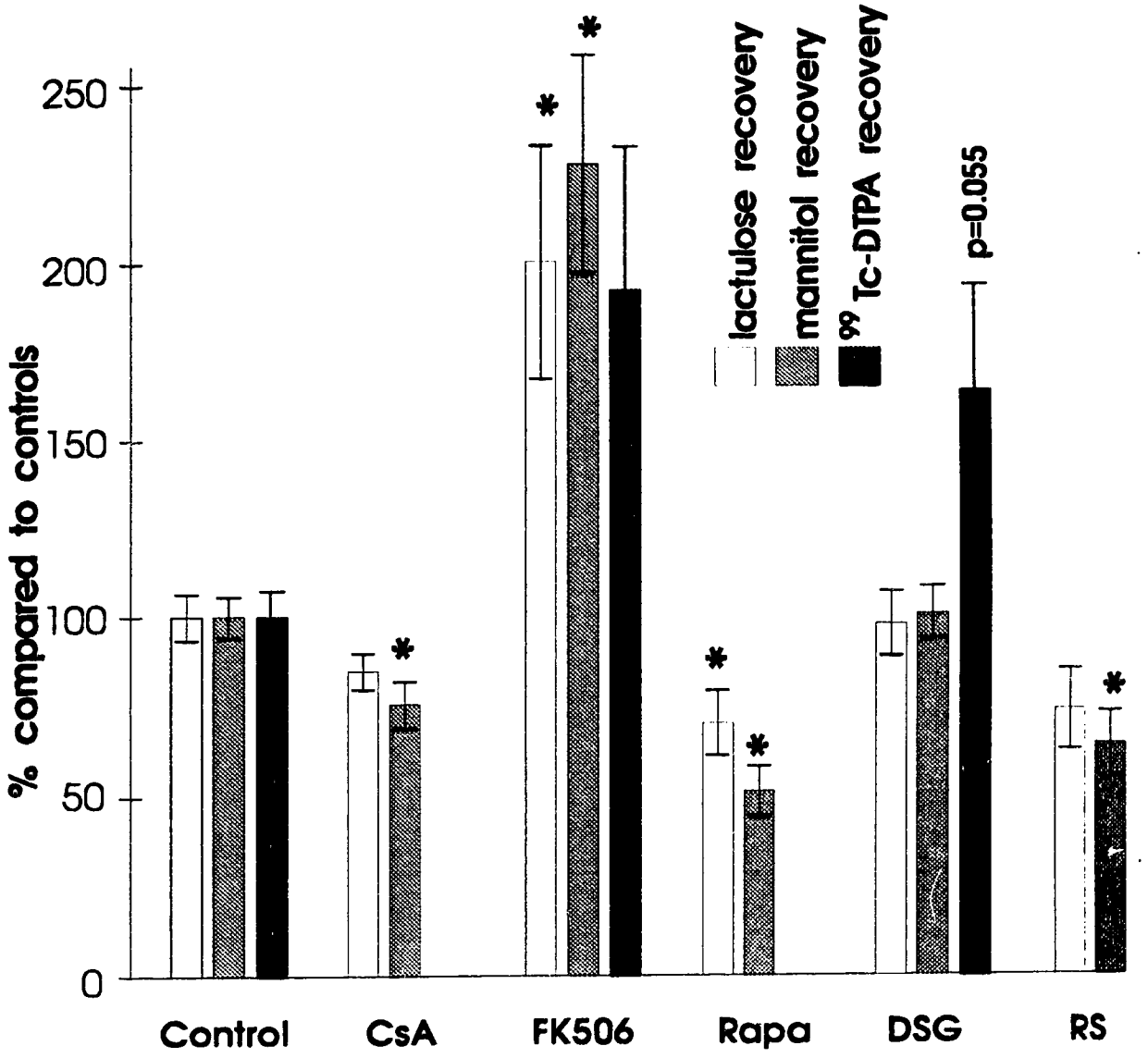


Values are means ± SEM

* significant difference compared to controls ($p<0.05$); compared with student's t-test.

Figure VIII-2

Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin and RS61443 on *In Vivo* Intestinal Permeability

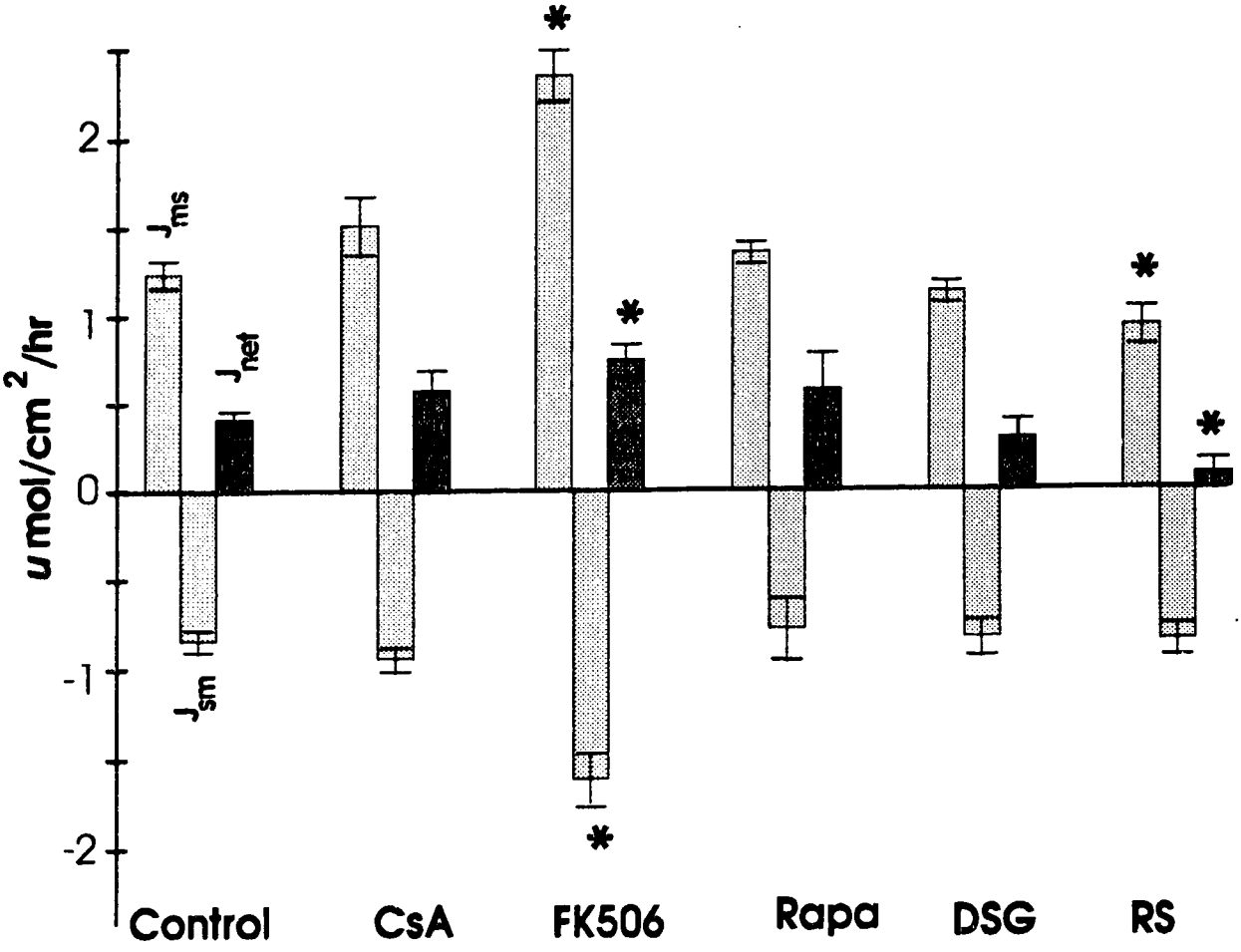


Values are means ± SEM

* significant difference compared to controls ($p<0.05$); compared with student's t-test.

Figure 3A

Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin and RS61443 on *In Vitro* 3-O-Me-Glucose Fluxes in the Jejunum

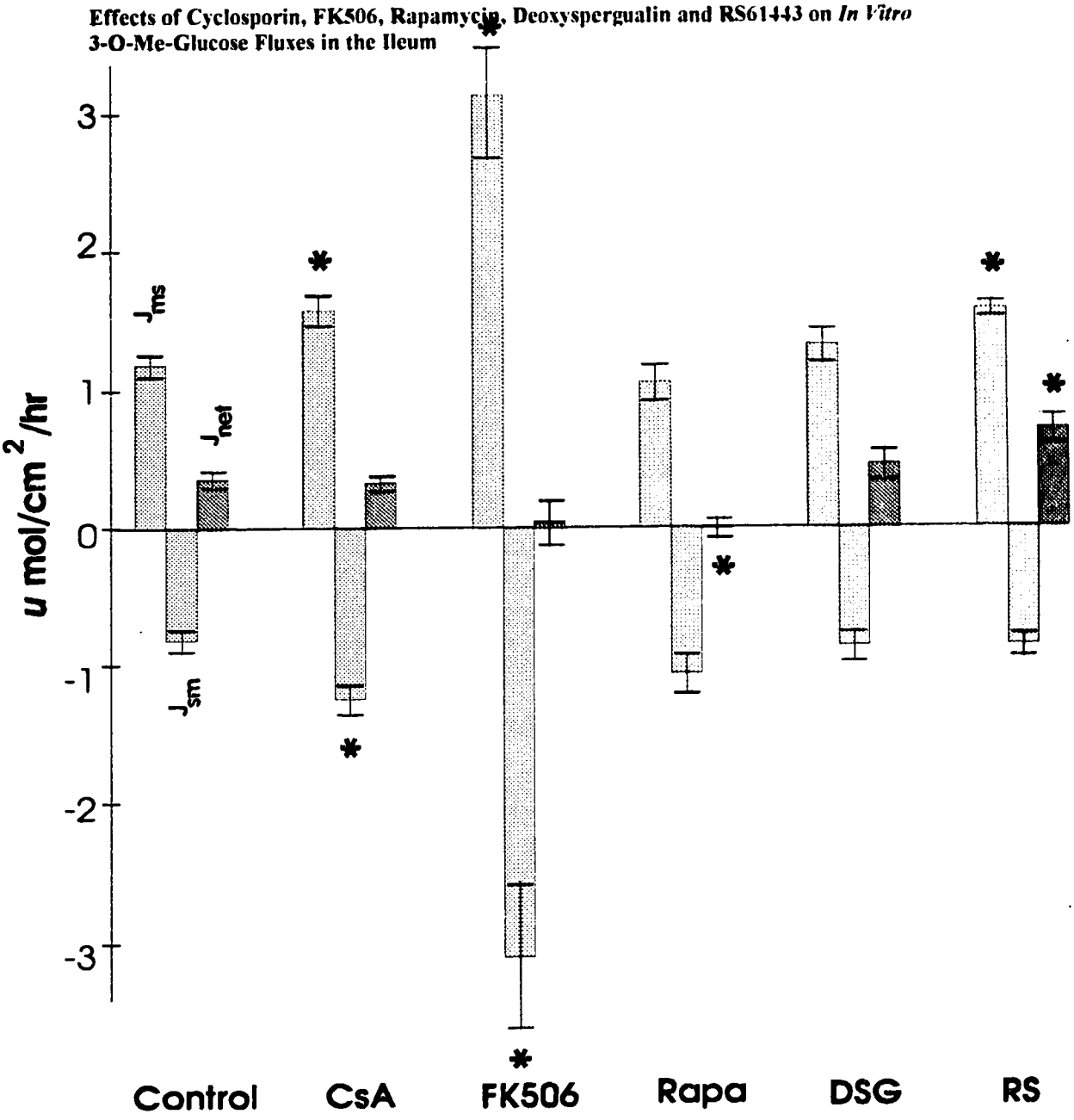


Values are means ± SEM

* significant difference compared to controls ($p<0.05$); compared with student's t-test.

Figure 3B

Effects of Cyclosporin, FK506, Rapamycin, Deoxyspergualin and RS61443 on *In Vitro* 3-O-Me-Glucose Fluxes in the Ileum

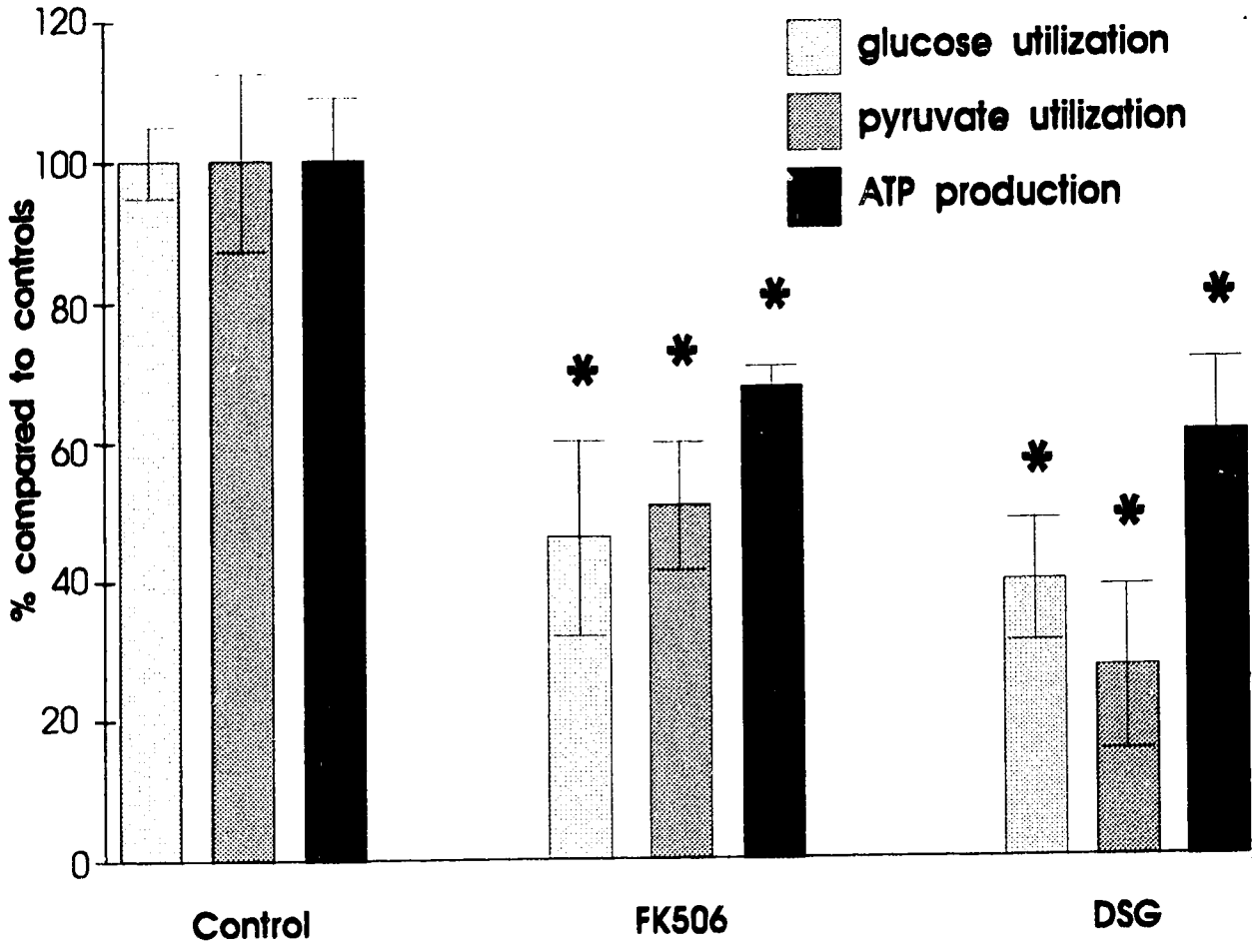


Values are means ± SEM

* significant difference compared to controls ($p<0.05$); compared with student's t-test.

Figure 4

Effects of FK506 and Deoxyspergualin on *In Vitro* Glucose and Pyruvate Utilization, and ATP Production by Enterocytes



Values are means ± SEM

* significant difference compared to controls ($p<0.05$); compared with student's t-test.

REFERENCES

1. Fischer JE. Metabolism in surgical patients: Protein, carbohydrate and fat utilization by oral and parenteral routes. In: Sabiston DC, Jr., editor. Textbook of Surgery, 14th ed. Philadelphia: W.B. Saunders Company, 1991: 103-140.

2. National Academy of Sciences. Nutrient requirements of laboratory animals. Washington, D.C., 1978.

3. Fischer JE. Metabolism in surgical patients: Protein, carbohydrate, and fat utilization by oral and parenteral routes. In: Sabiston DC, Jr., editor. Textbook of Surgery. Philadelphia: W.B. Saunders Company, 1991: 103-40.

4. Stone B, Warty V, Dindzans V, Van Thiel D. The mechanism of Cyclosporine-induced cholestasis in the rat. Transplant Proc 1988; 20 (suppl 3): 841-4.

5. Bohme M, Buchler M, Muller M, Keppler D. Differential inhibition by cyclosporins of primary-active ATP-dependent transporters in the hepatocyte canalicular membrane. FEBS 1993; 333(1,2): 193.

6. Collin J, Dennison AR, Watkins RM, Millard PR, Morris PJ. Segmental small intestinal allografts: II. Inadequate function with Cyclosporine immunosuppression: Evidence of a protein-losing enteropathy. Transplantation 1987; 44(4): 479-83.

7. Kadman M, Klunemann C, Bohme M, Ishikawa T, Gorgas K, Otto G, *et al.* Inhibition by cyclosporin A of adenosine triphosphate-dependent transport from the hepatocyte into bile. Gastroenterol 1993; 104: 1507-14.

8. Reese JC, Fairchild RB, Solomon H, Warty V, Kaminski DL. The effect of FK506 on canine bile flow. Transplantation 1993; 56(5); 1162-5.

9. Brems J, Reese J, Kane R, Kaminski DL. Effect of cyclosporine and steroids on canine bile flow. Hepatology 1991; 14: 523-7.

10. Jindal RM, Soltys K, Yost F, Beer E, Tepper MA, Cho SI. Effect of Deoxyspergualin on the endocrine fucntion of the rat pancreas. Transplantation 1993; 56(5): 1275-7.

11. Sigalet DL Kneteman NM, Thomson ABR. Reduction of nutrient absorption in normal rats by Cyclosporine. Transplantation 1992; 53(5):1103-7.

12. Heeckt PF, Halfter WM, Lee KKW, Schraut WH, Bauer AJ. Prolonged in vivo administration of cyclosporine causes enteric smooth muscle hyperplasia in normal rat intestine and small bowel grafts. In Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: P97.

13. Philpott DJ, Butzner JD, Meddings JB. Regulation of intestinal glucose transport. Can J Physiol Pharmacol 1992; 70: 1201-7.

14. Thomson ABR, Keelan M, Sigalet D, Fedorak R, Garg M, Clandinin MT. Patterns, mechanisms and signals for intestinal adaptation. Dig Dis 1990; 8: 99-111.

15. Karasov WH, Diamond JM. Adaptation of intestinal nutrient transport. In: Johnson LR, editor. Physiology of the gastrointestinal tract. 2nd ed. New York: Raven press 1987: 1489-97.

16. Fedorak RN, Cheeseman CI, Thomson ABR, Porter V. Altered glucose carrier expression: mechanism of intestinal adaptation during streptozocin-induced diabetes in rats. Am J Physiol 1991; 261(4): G585-G591.

17. Thomson ABR, Keelan M, Clandinin MT, Walker K. Dietary fat selectively alters transport properties of rat jejunum. J Clin Invest 1986; 77: 279-88.

18. Wysocki GP, Gretzinger HA, Laupacis A, Ulan RA, Stiller CR. Fibrous hyperplasia of the gingiva: A side effect of cyclosporin A therapy. Oral Surg Med Oral Pathol 1983; 55: 274-8.

19. Meddings JB. Membrane function: its relationship to intestinal absorption and malabsorption. Can J Gastroenterol 1990; 4: 39-46.

20. Chan P, Scoble JE, Senior JM, Varghese Z, Sweny P, Moorhead JF. Cyclosporin inhibition of glucose transport in cell culture. Transplant Proc 1989; 21(1): 922-3.

21. Sigalet DL, Kneteman NM, Simpson I, Walker K, Thomson ABR. Intestinal permeability after small intestinal transplantation and cyclosporine treatment. Transplant Proc 1992; 24(3): 1120-1.

22. Grant D, Lamaont D, Zhong R, Garcia B, Wang P, Stiller C, Duff J. ^{51}Cr-EDTA: A marker of early intestinal rejection in the rat. J Surg Res 1989; 46: 507-14.

23. Peters TJ, Bjarnason I. Uses and abuses of intestinhal permeability measurements. J Gastroenterol 1988; 2(3): 127-32.

24. Crane PW, Clark CL, Sowter C, *et al.* Microvascular changes induced by cyclosporine. Transplant Proc. 1991; 23: 487

25. Murray BM, Paller MS, Ferris TF. Effect of Cyclosporine administration on renal hemodynamics in conscious rats. Kidney Int 1985. 28: 767-74.

26. Kahan BD. Cyclosporin. N Engl J Med 1989; 321: 1725-38.

27. Pastorino JG, Snyder JW, Serroni A, Hoek JB, Farber JL. Cyclosporin and carnitine prevent the anoxic death of cultured hepatocytes by inhibiting the mitocondrial permeability transition. J Biol Chem 1993; 268(19): 13791-8.

28. Henke W, Jung K. Comparison of the effects of the immunosuppressive agents FK506 and cyclosporin A on rat kidney mitochondria. Biochem Pharmacol 1993; 46(5): 829-32.

29. Krentz AJ, Dousset B, Mayer D, McMaster P, Buckels J, Cramb R, *et al.* Metabolic effects of cyclosporin A and FK506 in liver transplant recipients. Diabetes 1993; 42: 1753-9.

30. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD, editors. Molecular biology of the cell. New York: Garland Publishing, Inc., 1983.

31. Mandel LJ, Bacallao R, Zampighi G. Uncoupling of the molecular 'fence' and paracellular 'gate' functions in epithelial tight junctions. Nature 1993; 361: 552-5.

32. Bjarnason I, Smethurst P, Macpherson A, Walker F, McElnay JC, Passmore P, Menzies IS. Glucose and citrate reduce the permeability changes caused by indomethacin in humans. Gastroenterology 1992; 102: 1546-50.

33. Somasundaram S, Macpherson AJ, Hayllar J, Saratchandra P, Bjarnason I. Enterocyte mitochondrial damage due to NSAID in the rat. Gut 1992; 33(suppl): S5.

34. Glanborg-JorgensenT, Weis-Fough US, Neilsen HH, Olsen HP. Salicylate and aspirin-induced uncoupling of oxidative phosphorylation in mitochondria isolated from the mucosal membrane of the stomach. Scand J Clin Invest 1976; 36: 649-53.

35. Bjarnason I, Hayllar J, Macpherson AJ, Russell AS. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology 1993; 104: 1832-47.

36. Wallemacq PE, Reding R. FK506 (Tacrolimus), a novel immunosuppressant in organ transplantation: Clinical, biomedical, and analytical aspects. Clin Chem 1993; 39(11): 2219-28.

37. Jayaraman T, Marks AR. Rapamycin-FKBP12 blocks proliferation, induces differentiation, and inhibits cdc2 kinase activity in a myogenic cell line. J Biol Chem 1993; 268(34): 25385-8.

38. Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell 1992; 69: 1227-36.

39. Francavilla A, Starzl TE, Scotti C, Carrieri G, Azzarone A, Zeng QH, *et al.* Inhibition of liver, likney, and intestine regeneration by Rapamycin. Transplantation 1992; 53(2): 296-8.

40. Yuh DD, Morris RE. The immunopharmacology of immunosuppression by 15-Deoxyspergualin. Transplantation 1993; 55(3): 578-91.

41. Sweeney MJ, Hoffman DH, Esterman MA. Metabolism and biochemistry of mycophenolic acid. Cancer Res 1972; 32: 1803-9.

42. Allison AC, Eugui EM. Inhibitors of de novo purine and pyrimidine synthesis as immunosuppressive drugs. Transplant Proc 1993; 25(3)(supp 2): 8-18.

43. Goulet O, Revillon Y, Brousse N, Colomb V, Jan D, Caniomi D, *et al.* Normal growth four and half years after small bowel transplantation using Cyclosporine A in an infant. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O7.

44. Grant D, Wall W, Mimeault R, Zhong R, Ghent C, Garcia B, *et al.* Successful small-bowel-liver transplantation. Lancet 1990; 335: 181-4.

45. Todo S, Tzakis A, Reyes J, Abu-Elmagd K, Casavilla A, Fung JJ, Starzl TE. Intestinal transplantation in humans under FK506. Transplant Proc 1993; 25(1): 1198-9.

46. Todo S, Tzakis A, Reyes J, Abu-Elmagd K, Furukawa H, Nour B, *et al.* Clinical intestinal transplantation: three-year experience. In: Abstracts of the IIIrd International Symposium on Small Bowel Transplantation; 1993 Nov; Paris: O2.

47. Tzakis AG, Reyes J, Todo S, Nour B, Shapiro R, Jordan M, *et al.* Two-year experience with FK506 in pediatric patients. Transplant Proc 1993; 25(1): 619-21.

48. Reding R, Wallemacq PE, Lamy ME, Rahier J, Sempoux C, DeBande B, *et al.* Conversion from cyclosporine to FK506 for salvage of immunocompromised pediatric liver allografts. Tranplantation 1994; 57(1): 93-100.

49. Chen H, Wu J, Xu D, Aboujaoude M, Stepkowski S, Kahan B, Daloze P. The effect of rapamycin on orthotopic small bowel transplantation in the rat. Transplant Proc 1992; 24(3): 1157-8.

50. Gannedahl G, Wanders A, Carlsson M, Tufveson G. 15-Deoxyspergualin effects in rat heart allograft transplantation--Relation to dose, timing, and cyclosporine. Transplantation 1993; 55(2): 455-6.

51. Gannedahl G, Wanders A, Carlsson M, Tufveson G. 15-Deoxyspergualin potentiates the immunosuppressive effect of Cyclosporine A in a rat heart allograft model. Transplant Proc 1993; 25(1): 768-9.

52. Gores PF, Najarian JS, Stephanian E, Lloveras J, Kelley S, Sutherland DER. Insulin independence in type I diabetes after transplantation of unpurified islets from single donor with 15-Deoxyspergualin. Lancet 1993; 341: 19-21.

53. D'Alessandro AM, Rankin M, McVey J, Hafez GR, Sollinger HW, Kalayoglu M, Belzer FO. Prolongation of canine intestinal allograft survival with RS-61443, Cyclosporine, and Prednisone. Transplantation 1993; 55(4): 695-701.

54. Platz KP, Sollinger HW, Hullett DA, Eckhoff DE, Eugui EM, Allison AC. RS-61443 -- a new, potent immunosuppressive agent. Transplantation 1991; 51(1): 27-31.

55. Morris RE, Hoyt EG, Murphy MP, Eugui EM, Allison AC. Mycophenolic acid morpholinoethylester (RS-61443) is a new imminosuppressant that prevents and halts heart allograft rejection by selective inhibition of T- and B-cell purine synthesis. Transplant Proc 1990; 22(4): 1659-62.

56. Bechstein WO, Schilling M, Steele D, Hullet D, Sollinger HW. RS-61443/Cyclosporine combination therapy prolongs canine liver allograft survival. Transplant Proc 1993; 25(1): 702-3.

57. Kahan BD, Tejpal N, Gibbons-Stubbers S, Tu Y, Wang M, Stepkowski S, Chou T-C. The synergistic interactions in vitro and in vivo of Brequinar Sodium with Cyclosporine or rapamycin alone and in triple combination. Transplantation 1993; 55(4): 894-900.

APPENDIX I

PLATES

Plate 1
Effects of Cyclosporin and RS61443 on Villus Morphometry in the Jejunum

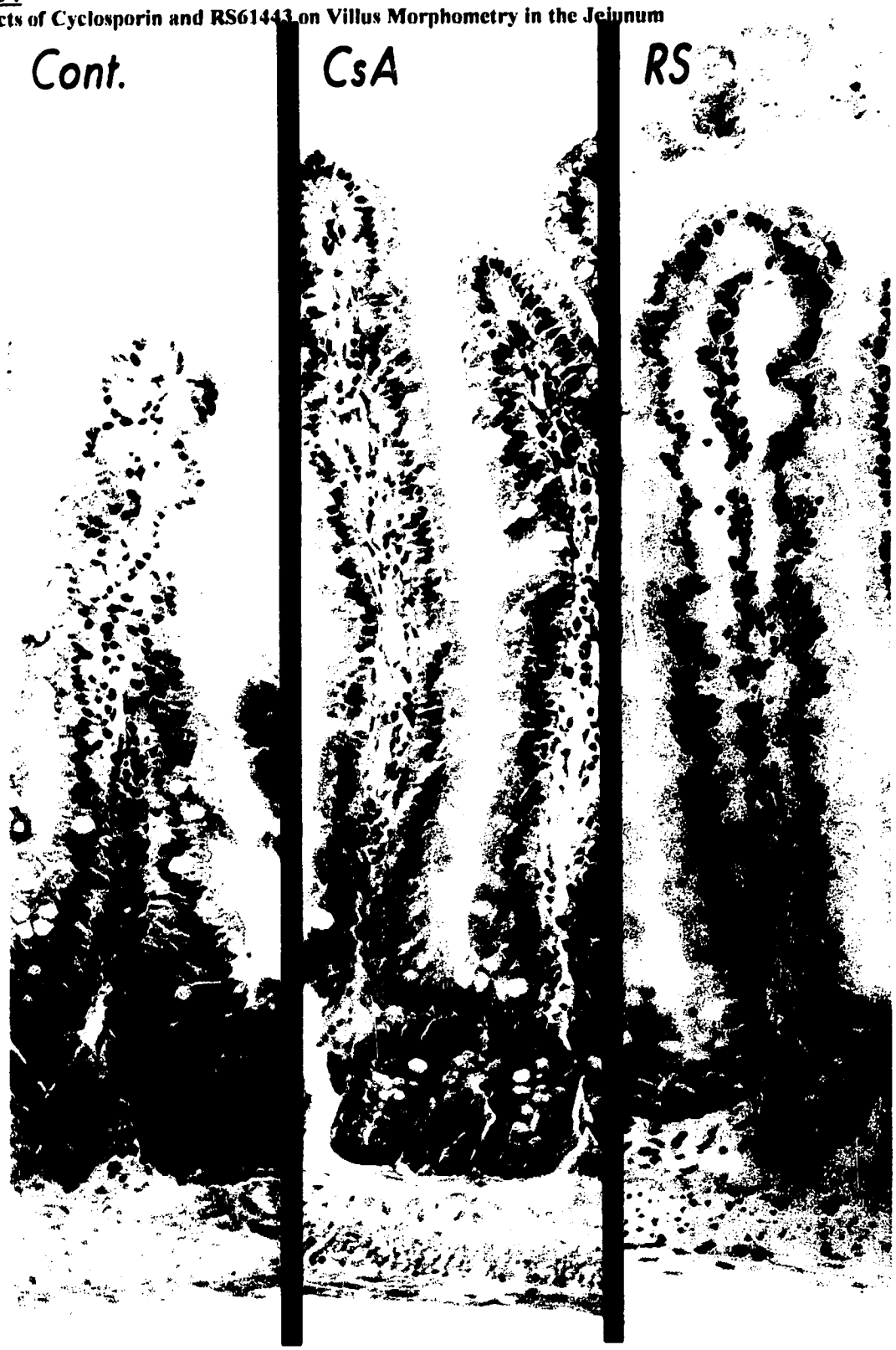
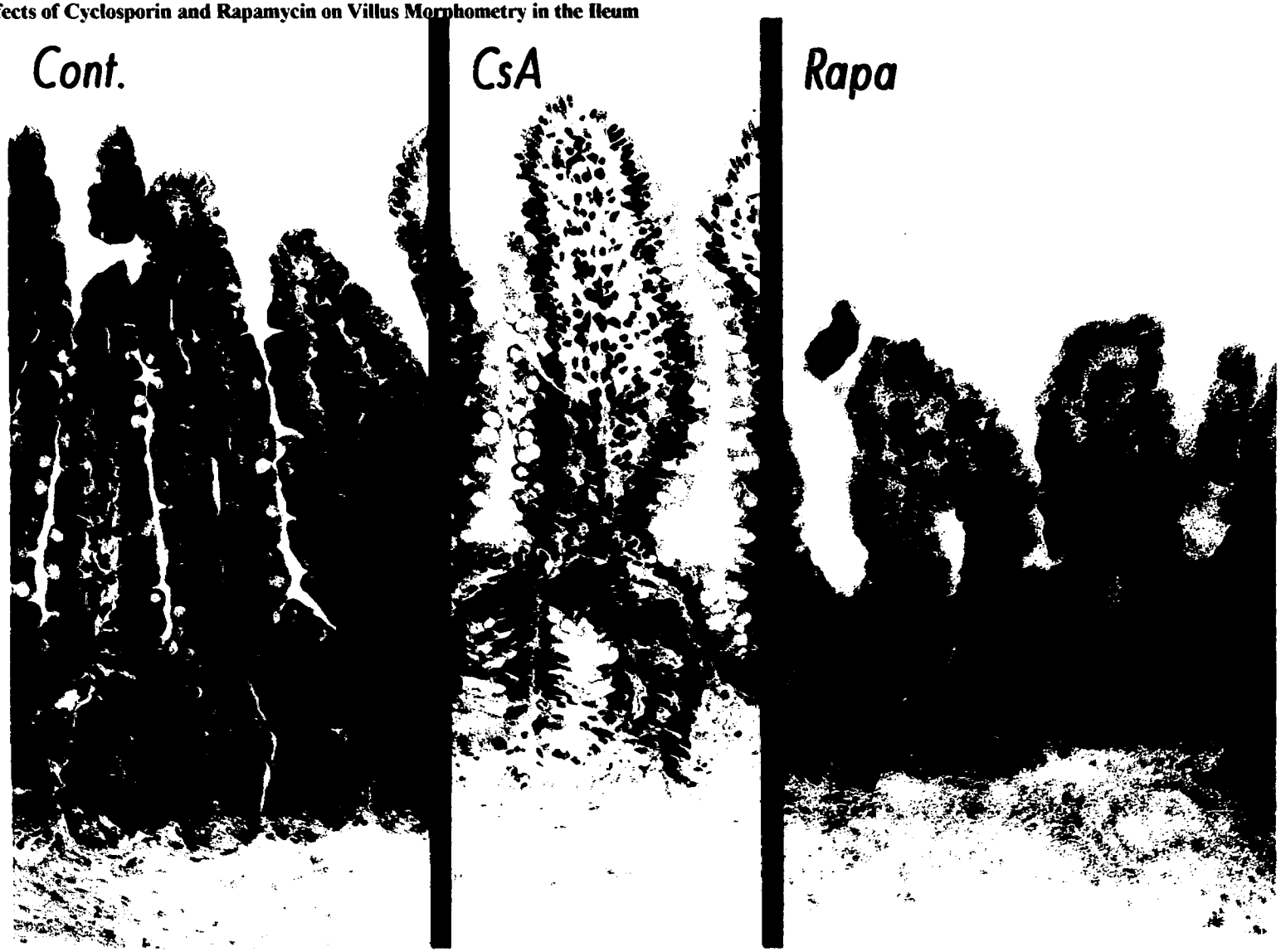


Plate 2

Effects of Cyclosporin and Rapamycin on Villus Morphometry in the Ileum



APPENDIX II

CALCULATIONS

CALCULATIONS OF ENERGY AVAILABLE FOR GROWTH

Constants
percentage water in feed = 1.0395

percentage of digestible energy (D.E.) which is metabolizable energy (M.E.) = 95% [1]

daily maintenance energy requirement for rats (based on 120% of basal metabolic rate) = E_M = $110/W^{0.75}$ in kcal, where W = weight in kg [1]

For a 300g rat, this is = to 44.6 kcal/day

energy content of dry feed = 4.7 kcal/g (determined from studies; data not shown)

Assume:
daily energy available for normal growth in a normal rat (controls) = E_G =
total energy absorbed (E_A)- maintenance energy (E_M)
and
E_A = daily feed intake in g/kg/d (FI) (Table VIII-1) x W ÷ 1.0395 x 4.7 kcal/g of feed x % energy absorption ($\%A_E$)/100 (Table VIII-1) x % of D.E. which is M.E.

Therefore,
$E_G = FI \times W \div 1.0395 \times 4.7 \text{ kcal/g} \times \%A_E/100 \times 0.95$

For our control rats:
E_G (in kcal) = 69.4 g/kg/d x W ÷ 1.0395 x 4.7 kcal/g x 81.3%/100 x 0.95 - $110/W^{0.75}$

Utilizing a standard rat weight of 300g and the values for FI and $\%A_E$ derived from Table VIII-1, the daily energy available for growth for the control and test animals are:

controls	28.1 kcal
CsA	30.6 kcal (↑ by 8.7% relative to controls)
FK506	23.5 kcal (↓ by 16.5% relative to controls)
RAPA	22.7 kcal (↓ by 19.3% relative to controls)
DSG	27.6 kcal (↓ by 1.9% relative to controls)
RS61443	31.3 kcal (↑ by 11.3% relative to controls)

CALCULATIONS OF ENERGY REQUIRED PER UNIT OF WEIGHT GAIN

If we wish to calculate the amount of energy normally needed per gram of weight gain, then,

Assume:
control animals are "normal"

Energy required for one gram of weight gain (E_W) =
$E_G \div$ rate of weight gain (g/d) (R_W)

Utilizing the values from Table VIII-1 and the results from the above calculations, the E_W for "normal" (control) rats =
$E_G \div R_W = 28.1$ kcal/d $\div$ 3.15 g/d = 8.92 kcal/g of weight gain.

Based on this value of $E_W = 8.92$ kcal/g, we would have expected the CsA-treated animals to have gained 30.6 kcal/d $\div$ 8.92 kcal/g = 3.42 g/d.

The actual weight gain, however, was only 2.95 g/d (from Table VIII-1) or 86% of expected.

These results, as well as those for the other test groups, are tabulated below:

	E_G (kcal)	**Expected Rate of weight gain (kcal/d) (calculated)**	**Actual rate of weight gain (kcal/d) (Table 1)**	**Actual weight gain as a percentage of expected**
Controls	28.1	3.15	3.15	100%
CsA	30.6	3.42	2.95	86%
FK506	23.5	2.63	0.65	25%
RAPA	22.7	2.54	0.84	33%
DSG	27.6	3.09	2.33	75%
RS61443	31.6	3.54	2.76	78%

CALCULATIONS OF ENERGY AVAILABLE DUE TO FAT ABSORPTION

Constants:
percentage water in feed = 1.0395

percentage of digestible energy (D.E.) which is metabolizable energy (M.E.) = 95% [1]

caloric content of fat = 9 kcal/g [2]

fat content of dry feed = 5.8% (determined from studies; data not shown)

Assume:
Energy available from absorption of fat in diet (E_F) =

FI x W ÷ 1.0395 x 5.8%/100 x 9 kcal/g of feed x %fat absorption (%A_F)/100 (Table VIII-1)

For our control rats:

E_F = 69.4 g/kg/d xW ÷ 1.0395 x 5.8%/100 x 9 kcal/g of feed x 76%/100

Utilizing a standard rat weight of 300g and the values for FI and %A_F derived from Table VIII-1, the daily energy available from the absorption of dietary fat for the control and test animals are:

controls 7.9 kcal

CsA 7.6 kcal (↓ by 3.4% relative to controls)

FK506 7.0 kcal (↓ by 11.6% relative to controls)

RAPA 7.1 kcal (↓ by 10.6% relative to controls)

DSG 7.0 kcal (↓ by 11.0% relative to controls)

RS61443 8.1 kcal (↑ by 2.9% relative to controls)

---

[1] National Academy of Sciences. Nutrient requirements of laboratory animals. Washington, D.C., 1978.

[2] Fischer JE. Metabolism in surgical patients: Protein, carbohydrate and fat utilization by oral and parenteral routes. In: Sabiston DC, Jr., editor. Textbook of Surgery, 14th ed. Philadelphia: W.B. Saunders Company, 1991: 103-140.

CURRICULUM VITAE

NATALIE LYNN YANCHAR, M.D., B.Sc.(Hons.)

7207-119 street
Edmonton, Alberta
T6G 1V9
Tel: (403) 432-0228 (home)
(403) 492-8822 (work)

EDUCATION

- B.Sc., Honors in Biochemistry, University of Alberta, Edmonton, Alberta, 1981-86.
- Doctorate of Medicine, Queen's University, Kingston, Ontario, 1986-90.
- Rotating Internship, Charles Camsell Provincial General Hospital, Edmonton, 1990-91.
- Residency in General Surgery, University of Alberta, 1991-present.
- Masters studies in Surgical Research, University of Alberta, supervisor, Dr. D.L. Sigalet, 1993-present.

ACADEMIC AWARDS/HONORS

- The Dean's Silver Medal in Science, University of Alberta, June, 1986.
- The Canadian Chemical Society Award (for highest standing in the University of Alberta biochemistry graduating class), June, 1986.
- Alberta Heritage Foundation for Medical Research (AHFMR) Summer Studentship Awards, 1982, 1984, 1986 & 1987.
- Honors standing in Surgery, Faculty of Medicine, Queen's University, 1990.
- The Sarah and Henry Scott Memorial Prize in Surgery, Queen's University, June, 1990.
- First Prize Award, Basic Science Research, Department of Surgery, University of Alberta, May, 1994
- Gregory C. Graham Award in Surgery, University of Alberta, July, 1994

RESEARCH EXPERIENCES

1982 - AHFMR Summer Studentship. Supervisor: Dr. J. Vederas, University of Alberta (U. of A). **Isolation of Cyclosporin A.** Involved methods of microbial fermentation followed by extraction and purification of cyclosporin A

1984 - AHFMR Summer Studentship. Supervisor: Dr. J.F. Henderson, U. of A. **Study and characterization of ATP levels in solid tumors.** Involved harvesting solid tumors from mice, developing methods of rapid tissue sectioning and ATP extraction, and subsequent analyses by HPLC and photometric assays.

1985 - Honors biochemistry 4th-year project. Supervisor: Dr. W. Paranchych, U. of A. **DNA sequencing and analysis of part of the pilin gene of *Pseudomonas aeruginosa*.**

1986 - Honors biochemistry 4th-year project. Supervisor: Dr. R. Hodges, U. of A. **Development of a synthetic peptide vaccine against *Influenza A*.** Involved automated peptide synthesis.

1986 & 87 - AHFMR Summer Studentships. Supervisor: Dr. W. Paranchych, U. of A. **Study of the FinO/P conjugation control system in the F and F-like plasmids.** Involved DNA isolation, cloning and sequencing, bacterial mating and genetic complementation studies, protein expression experiments, and development of assay protocols.

1990-1992 - General Surgery Research Project. Supervisor: Dr. J. Fischer, U. of A. **The spectrum of pediatric trauma received by the University of Alberta Hospital: rural vs. urban trauma.** Retrospective chart study. Presented at the 1993 Canadian Association of Pediatric Surgeons meeting, Victoria, September, 1993

1993-1994 - Masters in Experimental Research in Surgery. Supervisor: Dr. D.L. Sigalet, U. of A. **The effects of novel immunosuppressive agents on nutrition and small bowel function in normal rats.** Involving *in vivo* analyses of growth and nutrition in an animal model, *in vitro* nutrient uptake studies, small bowel histology and morphometry, an extensive literature review on the short bowel syndrome and small bowel transplantation, and attendance at the IIIrd International Symposium on Small Bowel Transplantation, Paris, November, 1993. Won first and second place standings in the category of Basic Science Research, at the Department of Surgery and Division of General Surgery research days, respectively, the University of Alberta, 1994.

PUBLICATIONS

- Frost, L.S., Lee, S., Yanchar, N. and Paranchych, W. ***finP* and *fisO* mutations in FinP anti-sense RNA suggest a model for FinOP action in the repression of bacterial conjugation by the F*lac* plasmid JCFLO.** Molecular and General Genetics, 1989, 218: 152-60.