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UNIVERSITY OF ALBERTA

NOVEL IMMUNOSUPPRESSIVE FACTOR IN HUMAN COLOSTRUM

BY

PADMA MANDALAPU

A thesis submitted to
the Faculty of Graduate Studies and Research in partial fulfillment of the
requirements for the degree of Master of Science.

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DEDICATION

To my loving parents.

ABSTRACT

Breast feeding of infants is beneficial not only for its unique nutritional value but also because of the important immunoregulatory influence it has on the immature immune system of the neonate. Studies comparing breast fed human infants with formula fed infants have shown enhanced immune responses within one month of birth in the breast fed infants, but little is known about the components which might be responsible for this activity.

In the present study, the effect of human colostrum on T cell immune functions in vitro has been investigated. Human colostrum contains an activity, termed Colostrum Inhibitory Factor (CIF), that inhibits the induction of Interleukin 2 (IL 2) in T cell lines. Colostrum at 10% by volume was not cytotoxic to any of the cell lines tested and did not affect the proliferation of autonomously growing cells. CIF was specific to colostrum and was not found in mature milk. In PMA-stimulated EL4 T cells, inhibition is the result of blocking the accumulation of IL2 mRNA. Defatted, cell free human colostrum contained in the order of 100 U/ml of CIF activity, where 1 U/mL inhibits 50% of the IL2 response. The CIF activity increased by 2 to 4-fold following transient acidification to pH 3.0. On gel exclusion chromatography the inhibitory activity eluted in fractions corresponding to high molecular weight (over 150,000). Although CIF resembles TGFβ in some respects, antisera against TGFβ neutralized 10% or less of the CIF activity in human colostrum, whether acid-activated or not. CIF also behaved differently from TGFβ-1 and -2 in its mode of inhibiting IL2 production. Whereas TGFβ-1 and -2 blocked the induction of IL2 in EL4.E1 cells stimulated with PMA almost completely, they inhibited only about 50% of the response when EL4.E1 cells, or Jurkat cells, were stimulated with the combination of PMA and a calcium ionophore. CIF, on the other

hand, inhibited IL2 production completely, even when PMA and ionomycin were used. Thus, CIF differs both antigenically and functionally from TGFβ.

To futher characterize CIF, bovine colostrum was studied. A similar CIF activity was also detected in bovine colostrum, but not in normal bovine milk. Bovine F2 has 400 U/ml of activity. Like human F2, bovine F2 had increased activity on acidification and a significant portion of this activity was not blocked by panspecific anti TGFβ antibodies. The activity in bovine colostrum was concentrated in the casein fraction. On dissolution of casein micelles with EDTA and reprecipitation with calcium, the activity remained associated with casein. The present study, therefore, suggests the presence of a novel immunosuppressant factor in human and bovine colostrum that inhibits the production of IL2 and does not belong to any of the known TGFβ group of immunosuppressants. The activity coprecipitates with casein. The function of such immunosuppressant factors in colostrum is not clear.

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Padma Mandalapu

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LIST OF ABBREVIATIONS

BCG	Bacillus calmette guerin
BSA	Bovine serum albumin
CBG	Corticosteroid binding globulin
CD	Cluster of differentiation
CIF	Colostrum inhibitory factor
Con-A	Concanavalin A
CsA	Cyclosporine A
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleuside triphosphates
DTT	Dithiothreitol
EGF	Epidermal growth factor
F2	Fraction 2 (defatted, cell free colostrum)
F2A	Fraction 2 acidified
IDDM	Insulin dependent diabetes mellitus
IL	Interleukin, such as Interleukin 2
IFNγ	Interferron gamma
MLR	Mixed lymphocyte reaction
MOPS	3-[N-morpholinol] propanesulfonic acid
MTL	Mouse cytotoxic T lymphocyte line
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NF-AT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PHA	Phytohaemagglutinin
PMA	Phorbol-12-myristate-13-acetate
PPD	Purified protein derivative
rRNasin	Recombinant ribonuclease inhibitor
TBS	Tris-buffered saline
TGFβ	Transforming growth factor beta

1. INTRODUCTION

1. 1 General Introduction

Over the last decade there has been a growing consensus that breast milk provides optimal feeding for the newborn with well established nutritional advantages (Welsh et al 1979, Cunningham et al 1991). Human milk has many unique properties clearly beneficial for the neonate, since it contains biochemical components (Lonnerdal et al 1985) and immunological factors which satisfy their nutritional requirements and augments their defense mechanisms (Goldman et al 1973, Pittard et al 1979).

Immediately after birth, the newborn transits from the sterile conditions of the uterus into the unsterile environment. The immature immune system initially depends on maternal help, particularly when host defenses are considered. Multifunctional factors contained in maternal colostrum and milk augment the newborn's resistance to bacterial and viral infections and promote the harmonious development of the bacterial flora of the gut (Faden and Ogra 1981). Breast feeding and oral administration of colostrum has been found to confer a significant degree of immunity against a number of naturally acquired or vaccine-induced enteric and upper respiratory infections (Howie et al 1990, Frank et al 1982). This protection appears to be mediated largely through the secretory IgA antibody in colostrum. Nonspecific factors like lactoferrin, lysozyme, lactoperoxidase and bifidus factors play important bacteriostatic and bacteriocidal roles . In addition, suckling neonates are benefited by the supply of several types of growth promoting factors among which are epidermal growth factor, insulin, somatomedin and transforming growth factor β (TGFβ) (Grosvenor et al 1993). Growth factors in milk have the potential to stimulate proliferation and differentiation in lymphoid tissue and they may be an important component of the protection conferred by breast feeding, particularly in the early neonatal period. The soluble fraction of colostrum has in addition a significant concentration of

cytokines such as interleukin-1 (IL1) (Munoz et al 1990), tumor necrosis factor (Rudloff et al 1992) and interleukin-6 (Saito et al 1991) which are important mediators of the acute response to microbial invasion, inflammation and tissue injury. These cytokines have several important immunomodulatory properties. Bocci et al (1991), have suggested that although most of the cytokines ingested during the first six months of life are inactivated either in the stomach or the gut, a significant portion binds to the oro-pharyngeal lymphoid tissue and favours the maturation of the immune system by amplification mechanisms.

The potential immunological benefits of breast feeding of human infants was illustrated in a variety of studies. In one study breast fed infants (Pabst et al 1989) of Canadian native mothers developed a stronger cell mediated response to BCG when immunized at birth, than did formula fed infants. In another study breast fed children of Caucasian mothers (Pabst and Spady 1990) immunized at 2, 4, and 6 months with conjugate Haemophilus influenzae type 'b' vaccine had a higher antibody level when compared to a formula fed group at 7 and 12 months. Other studies have shown transmission of cellular hypersensitivity to Schistosoma mansoni by breast feeding (Eissa et al 1989). Breast feeding has also decreased the risk of childhood cancer (Davis et al 1988). Compared with breast feeding for more than 6 months, a raised risk for total cancer was found in infants breast fed for less than 6 months or fed by artificial formula . In this regard Davis et al have postulated that ineffective immunoregulation of activated B and T lymphocytes can lead to increased incidence of tumors in non breast fed infants Immunoregulation may be altered in some children who were not breast fed during their early exposure to viral infections. Mayer et al (1988) have shown that breast feeding can provide protection against the development of insulin dependent diabetes mellitus (IDDM). They suggested that breast milk could be the vehicle for the transmission of a specific agent that ultimately prevents pancreatic β cell destruction. Koletzko et al (1989) have shown that breast fed infants have a decreased incidence of Crohn's disease. In this

regard they suggested that Crohn's disease may develop in susceptible people as result of an immunological response to an unidentified antigen in the mucosa. Such an immunoregulatory abnormality may be influenced by breast feeding practices in early infancy. These studies indicate that breast feeding, in addition to providing temporary protection to the child, may also be important for the establishment of immunological parameters perinatally, to insure proper functioning of the immune system in later life.

Colostrum contains a variety of immunomodulatory factors, some of which are well known. For example, in addition to cortisol (Kulski et al 1981), which is immunosuppresive, colostrum contains members of the TGFβ family of cytokines (Saito et al 1993, Noda et al 1984). A mitogenic activity that acts on fibroblasts (Tapper et al 1979) and a proline rich protein that stimulates the proliferation of B lymphocytes have been described (Julius et al 1988). Human colostrum also contains both stimulatory factors and factors that inhibit T cell growth (Mincheva-Nilsson et al 1991). A factor that inhibits the production of IL2 has been documented (Hooton et al 1991). Such immunosuppressive factors in colostrum may have an important influence on the regulation of the immature immune system of the neonate. They might play on important role in preventing autoimmune diseases such as IDDM, or chronic inflammatory diseases such as Crohn's disease. They appear to be associated with decreased incidence of allergies in breast fed babies (Bocci et al 1991). Such factors may enable the infant to distinguish between self and non-self more effectively than would otherwise be possible.

This study is a continuation of Dr. Hooton's findings and involves the characterization of immunosuppressive factors that are specific to colostrum and not found in mature milk.

1.2 Biochemistry of Colostrum and Milk

Human milk contains a unique mixture of proteins, carbohydrates, fats, hormones, trace elements, and vitamins. The protein components of breast milk includes caseins, whey proteins, fat globule associated proteins, immunoglobulins, enzymes, mineral binding proteins, and cellular proteins. These proteins have varying physiochemical properties, functions and nutritional significance. Human milk also contains several nitrogenous compounds that may potentially be utilized for protein synthesis (such as peptides, free amino acids, and amino sugars), in energy metabolism (such as carnitine and choline) and for amino acid synthesis (such as urea and ammonia). Nonprotein nitrogen accounts for approximately 25% of the total nitrogen in human milk. Nucleotide nitrogen accounts for 0.4% to 0.6% of nonprotein nitrogen . Nucleotide's contribute to the enhanced immunity of the breast fed infant by significantly increasing IL2 production by stimulated mononuclear cells and enhances natural killer cell cytotoxicity (Carver et al 1991).

Casein is the major protein in the milk of most species comprising several phosphoproteins of molecular weight 26,000 to 45,000. Human milk is exceptional in having a low casein concentration, only about 20% of the total protein (Jenness 1979). The concentration of casein is three to four times higher in human colostrum compared to mature milk. Table 1.1 summarizes the values of total protein and casein concentration in bovine and human colostrum and milk (Larson 1985, White 1973).

Table 1.1: Total Protein and Casein Concentrations in Human and Bovine Milk and Colostrum.

	Protein (gm/L)	Casein (gm/L)
Bovine milk	33	26
Bovine colostrum	176	80
Human milk	9-12	2.5-4
Human colostrum	27	5-10

In the presence of millimolar concentration of calcium and phosphate casein molecules associate to form casein micelles (Farrell et al 1976, McMahon et al 1984). This unique protein aggregate, with a diameter of about 140 nanometers, contains up to 25,000 monomeric casein molecules (Jenness 1979). Casein appears to be a highly efficient package for the delivery of protein and salts to the infant. Human milk has a ten fold lower casein content and a higher non-protein nitrogen content than bovine milk. Human casein is more tightly associated with whey proteins, and contains considerably more carbohydrate components and less phosphate than bovine casein (Blanc 1981).

Casein consists of several proteins , αs1-, αs2-, β-, and κ-caseins which are associated into micelles in milk. Bovine casein contains 45% α–casein, 30% β-casein, and 15% κ-casein; human casein which contains 20% κ–casein differs by its high level of β–casein (50%) in contrast with its low content of α-casein (10%) (Samour et al 1988). Enzymatic digestion of casein yields numerous short peptides with biological activities. β–casein in particular generates active peptides such as opiate-like β-casomorphins, morphiceptin and immunostimulating peptides all of which are associated with the hydrophobic part of the molecule. These peptides stimulate the phagocytic activity of macrophages and might play a role in the proliferation and maturation of T cells and natural killer cells for the defense of the neonate against infectious agents (Samour et al 1988). Several homologous major whey proteins are present in milks of all species. Their concentrations in bovine and human milk are summarized in Table 1.2 (Larson 1985).

The carbohydrates in milk consist of mono-, di- and oligosaccharides as well as glycoproteins and glycosphingolipids. In human milk carbohydrates constitute about 40% of the energy consumed during the nursing period (Blanc 1981). The disaccharide lactose is the major sugar in milk and is synthesized within the Golgi secretory system of the mammary alveolar cell (Brew et al 1975). Human milk contains approximately 6-7g

lactose/100 ml milk and bovine milk has 4.7g/100 ml milk (Blanc 1981). Glucose and galactose are the major monosaccharides (Ebner et al 1974).

Table 1.2: Proteins in Human and Bovine Milk (mg/ 100 ml)

Proteins	Human	Bovine
α - Lactalbumin	250	125
β - Lactoglobulin	0	300
Lactoferrin	175	trace
Lysozyme	45	trace
Serum Albumin	50	50
IgA	125	7
IgG	4	45
IgM	3	7
Free Secretory Component	unknown	5
Caseins	250	2800
Total Protein	900	3500

Milk lipids are a major source of concentrated high energy substrate for most young mammals, supplying about 4.9 cal/gm of milk fat (Neville 1983). Human milk contains about 4% of fat which supplies 40% of the calories (Lemons et al 1980). About 98% of the lipid in milk is triglyceride contained in the membrane bound fat droplet called milk fat globule. Other lipid constituents of milk include cholesterol, phospholipids, vitamin A, vitamin D, E and a large number of minor lipids. Milk fat content and components are affected by diet. Human milk also contains long chain polyunsaturated essential fatty acids which are useful for the synthesis of complex structural lipids and prostaglandins (Clandinin et al 1985).

The trace element content of milk is well balanced compared to many foods, making milk a high quality source of these nutrients. The chief trace elements in milk are iron, zinc, iodine, selenium, sulfur, and cobalt. The bioavailability of these elements is high in human milk when compared to bovine milk.

1.3 Hormones and Growth Factors in Human Milk

Human milk and colostrum contain a variety of proteins, peptides and steroids that posses biological activity (Grosvenor et al 1993). The concentration of these hormones and growth factors is generally higher in colostrum than in later milk. In addition their concentration in milk exceeds that of the maternal serum. Some of these hormones are synthesized within the mammary gland and others are being transported from the maternal circulation. Grosvenor et al have suggested three important functions of these hormones and growth factors which are as follows:

1. The mammary gland could be viewed as an excretory gland for the transport of these biologically active substances via colostrum and milk.
2. These substances may influence the growth and function of the mammary gland.
3. They may function in the regulation of growth and differentiation of various neonatal tissues.

Kidwell and Salomon 1989 have suggested that epidermal growth factors (EGF) in milk and colostrum may produce biological effects on non-gastrointestinal targets in the neonate. Fetal and neonatal tissues (gut and lung) possess EGF receptors and in culture respond mitogenically to EGF.

The various hormones and growth factors in milk are summarized in Table 1.3. The concentrations of glucocorticoids in milk are lower than in plasma (Grosvenor et al 1993). Cortisol in colostrum averaged 7.5% of that found in serum during late pregnancy. Cortisol was relatively high in mammary secretions during late pregnancy (25.5 ± 18 ng/ml) and decreased within two days postpartum (10.2 ± 2.0 ng/ml) to reach

1.8 ± 0.7 ng/ml by ten days postpartum (Kulski et al 1981). These authors have suggested that cortisol in breast milk may help to control the transport of fluids and salts out of the incompletely developed gastrointestinal tract of infants.

Cortisol in milk binds to corticosteroid binding globulin (CBG), albumin and membranes of fat globules. Tucker et al (1977) have suggested that these binding proteins may be involved in the transport of cortisol from blood into mammary secretions. The postpartum fall in cortisol values is related to the changes in the concentration of CBG and albumin, which are high in colostrum and then decrease rapidly two to three days after delivery (Rosner et al 1975). Significant concentrations of other hormones such as estrogens and progesterones have been detected in milk.

Growth factors like epidermal growth factor and transforming growth factors in milk are considered to influence the immune system and the growth of the neonate. The concentration of these growth factors is higher in colostrum compared to mature milk. Transforming growth factor β's (TGFβ) are polypeptides that may influence the growth of a variety of cell types in a positive or negative fashion. They are produced as latent, high molecular weight complexes which are activated on transient acidification, heating, or enzymatic treatment with plasmin, cathepsin or glycosidases (Miyazono et al 1991). In colostrum the total amount of TGF β is 1370 ± 240 ng/ml of which the active form comprised 730 ± 250 ng/ml. In late milk the total TGF β is 955 ± 210 ng/ml, with an active form of 180 ± 155 ng/ml (Saito et al 1993). These authors have suggested that both TGF β1 and TGF β2 exist in human milk and colostrum. Significant concentrations of other growth factors such as insulin like growth factor, mammary derived growth factor, fibroblast growth factor etc., have been identified in milk. In addition, inhibitors of growth have been detected in milk, for example mammostatin, a polypeptide purified from normal human mammary epithelial cells, which blocks DNA synthesis in culture

Table 1.3. Hormones and Growth Factors in Human Milk. (Grosvenor 1993)

Hormones	Growth factors
A. Adrenal gland hormones	A. Insulin-like growth factors (IGFs)
B. Gonadal hormones	B. IGF binding proteins
1. Estrogens	C. EGF and Transforming growth factor α
2. Progesterone	
C. Brain - gut hormones	D. Transforming growth factor β
1.GnRH	E. Other growth factors
2.Somatostatin (SS)	F. Growth inhibitors in milk
3.GH-releasing hormone	
4.TRH	
D. Growth hormones	
1. Insulin	
2. Relaxin	
3. GH	
4. Prolactin (PRL)	
E. Other hormones	
1. Calcitonin (CT)	
2.PTH-related peptide	
3. Erythropoietin	
4. Thyroid gland hormones	
F. Prostaglandins	

and transforms mammary epithelial cells. A 13 kD polypeptide termed mammary derived growth inhibitor (MDGI) has been purified from bovine mammary tissue and from milk fat globule, that inhibits the proliferation of several normal and transformed mammary epithelial cells or cell lines in vitro (Grosse et al 1993). Prostaglandins E and F have been identified in human and bovine milk. They exert a cytoprotective effect on the intestine of the nursing neonate. Grosvenor et al (1993) have suggested that these hormones and growth factors may have both short and long term effects on the neonate. On a short term basis they effect the endocrine and metabolic functions and long term effects are seen on the immune, gastrointestinal and neuroendocrine system.

1.4 Humoral Anti-Infective Components in Breast Milk

Breast fed infants have lower rates of morbidity and mortality related to infections than formula fed infants (Hanson et al 1972). Protective factors in human milk that have the potential to modify or prevent infections may be cells, non-antibody factors and antibodies. Colostrum in general contains higher concentrations of protein, lactoferrin, and immunoglobulins but lower concentrations of fat and lactosethan later milk. The repertoire of antibodies found in human milk is extensive, with more than 20 viral, bacterial, and toxin-specific antibodies. Secretory IgA is a major immunoglobulin in human milk. Although appreciable amounts of 7S (serum type) IgA, IgG, and IgM classes of immunoglobulins are found in colostrum and milk, the bulk of the immunoglobulin in colostrum is secretory (11S) type IgA. Among the subclasses of IgG, the percentage of IgG1 is significantly increased and that of IgG2 is decreased in both colostrum and milk relative to the percentage distribution reported in maternal sera and other healthy adults (Mehta et al 1989).

Colostrum contains up to 5 mg/ml IgA, which decreases in mature milk to 1 mg/ml. Secetory IgA is quite resistant to pH changes or proteolytic enzymes, and functions by binding antigens such as microorganisms and non-degraded food proteins, preventing them from reaching the mucosal membranes where they might initiate

infection or inflammation. The nonantibody antibacterial protective factors present in human milk are summarized in Table 1.4 (Pickering et al 1986).

In addition to the antibacterial protective factors, human milk has several nonantibody antiviral, and antiprotozoal protective factors. Lipids in milk have an important role as antiviral factors. Unsaturated fatty acids and monoglycerides inactivate enveloped viruses including herpes simplex, Semliki Forest, influenza, and Ros River virus (Pickering et al 1986). Many unidentified macromolecules play an important role in viral infections by inhibiting virus attachment and penetration. Alpha-1-antitrypsin in human milk has a protective role against rotavirus infection in breast fed infants. Bile salt stimulated lipase inactivates viruses and bacteria by generating fatty acids and monoglycerides . Protozoans like Giardia lamblia and Entamoeba histolytica are rapidly killed by exposure to human milk. This protective effect is in part due to bile salt stimulated lipases in milk (Gillen et al 1983).

1.5 Cellular Components of Human Milk

In all species studied viable lymphocytes and macrophages are normal components of colostrum and milk. Cell concentration and predominant cell type vary with duration of lactation. Colostrum secreted from days one through four postpartum, normally contains 10^5 to 5 x 10^6 leukocytes/ml, with approximately 70 to 100% of these being phagocytes (30 to 50% mononuclear phagocytes and 40 to 60% polymorphonuclear cells). Mature milk, after four days lactation, typically contains fewer cells, in the range of 10^5 /ml with mononuclear phagocyte predominance, macrophages comprising 50 to 80% of these cells. Beyond four to six weeks of lactation, milk phagocyte numbers fall, being almost totally replaced by scant numbers of epithelial cells and cell like particles (Buescher et al 1986, Crago et al 1979).

Table 1.4: Nonantibody, Antibacterial Protective Factors in Human Milk

Factors	Proposed Mechanisms of Action	Organisms Effected
bifidus factor	inhibits replication of certain bacteria in the gastrointerstinal tract by causing a proliferation of lactobacilli	Enterobacteriaceae including shigella, salmonella, and some Escherichia coli
complement components	opsonic, chemotatic and bacteriolytic activity	E.coli
lysozyme	with IgA, peroxide or ascorbate, causes lysis of bacteria	E.coli Salmonella
lactoferrin	binds ferric iron	E.coli Candida albicans
lactoperoxidase	oxidation of bacteria	E.coli Salmonella typhimurium
nonantibody proteins: receptor-like glycolipid or glycoprotein	inhibit bacterial adherence	V.cholerae
gangliosides	interfere with attachment of enterotoxin of GM_1 cell membrane ganglioside receptors	E.coli and V. cholerae enterotoxins
nonlactose carbohydrate factors	prevent action of stable toxin	E.coli ST

Lymphocytes are a quantitatively minor subset usually constituting less than 10% of colostral cells. Fifty percent of colostral lymphocytes are T cells and the great majority of these display the phenotypic and functional characteristics of memory T lymphocytes. They exhibit the CD45Rlow, CDw29, UCHL1, LFA-1high memory T cell phenotype (Bertotto et al 1991). These cells are able to proliferate in response to anti-CD3 and anti-CD2 monoclonal antibodies and produce interferon-gamma, similar to autologous and heterologous blood samples. Colostral T cells are mostly delta-TCS-1+ similar to that of the intestinal intraepithelial counterpart, suggesting that they are actively motile cells capable of migrating from lymphoid to extra lymphoid body tissues. The gamma/delta T cells in colostrum have a phenotypic pattern similar to that of intestinal intraepithelial cells. These findings suggest that these cells might originate in the gut-associated lymphoid system and home selectively to the mammary gland late in pregnancy and throughout lactation (Bertotto et al 1991).

About 34% of colostral lymphocytes are B cells. Fifty percent of the colostral B cells bear IgA on their surface (Diaz-Jouanen et al 1974). The viable lymphocytes in colostrum and milk undergo blast transformation and proliferation when exposed to mitogens or cellular alloantigens, but are not responsive to certain microbial antigens that stimulate blood lymphocytes from the same individuals. These findings again suggest that the breast is a site to which selected population of lymphocytes home (Parmely et al 1977). These authors have suggested that milk lymphocyte unresponsiveness is an antigen specific phenomenon that reflects the absence of certain antigen reactive clones in milk in contrast to those present in blood . Keller et al (1981) and Nikolova et al (1990) have shown that colostral cells are immunologically competent and possibly important effector cells in neonatal immunity. They have shown that mitogen stimulated milk lymphocytes are capable of producing lymphokines such as IL1, IL2, IFNγ and lymphocyte derived chemotactic factors.

Various studies have shown transmission of T cell mediated immunity from mother to neonate to various infectious agents. In one study, female F1 rats infected with Trichinella spiralis were able to transfer immunity to their suckling offsprings (Kumar et al 1989). Oral feeding of sensitized T lymphocytes to newborn calves can lead to functional immunity against rotavirus infection (Archambault et al 1988). Neonates given primed T lymphocytes orally early in lactation and prior to challenge with the organisms were rendered immune. Schlesinger et al (1977) have shown the transmission of lymphocyte responses to tuberculin by breast feeding. Riedel-Caspari et al (1991) have shown that calves fed with milk supplemented with colostral cells have a higher blastogenic response to concanavalin A and show enhanced antibody production against sheep erythrocytes. It has also been demonstrated in animal studies that cellular components in colostrum and milk are immunologically active and remain viable within the gastrointestinal tract of the neonate (Vidyasagar et al 1989, Seelig et al 1987). The sensitized lymphocytes in colostrum and milk can originate from extramammary sites, with both peripheral and mesentric lymphocytes homing to the mammary gland and milk (Manning & Parmely 1980). Parmely et al (1976) found that milk T cells can recognize foreign antigens and respond as well as blood lymphocytes to histocompatibility antigens. Other antigens to which breast milk lymphocytes have been reported to respond include E coli K1 (Ogra et al 1979) and PPD, while response to candida antigens was not found (Parmely et al 1976). In this regard, these authors have suggested that there appears to be a homing process in the mammary glands which selects specific T cells. It has also been shown by Parmely and William (1979) that there is a control mechanism of cytotoxic T lymphocytes in the mammary gland especially for those specific for paternal antigens.

1.6 Immunological Benefits of Breast Feeding for the Baby

1.6.1 Protection against Bacterial Infection

Resistance to infection and protection from allergic disorders are the most frequently cited immunologic benefits of breast feeding. A variety of studies have shown

that the morbidity and mortality of breast fed babies are lower than that of formula fed babies (Cunningham et al 1979, Goldsmith et al 1973). In all populations, breast feeding appears to protect most effectively against diarrhea, followed by upper respiratory tract infections. Factors that play an important role in the prevention of diarrhea in exclusively breast fed babies are the initial establishment of a nonpathogenic gut flora and subsequent avoidance of pathogens which might colonize the gut. Lactobacilli and bifidus factor found in the gut of breast fed babies help to maintain a pH of 5 to 6. This low pH inhibits the growth of bacteria such as Streptococcus fecalis, Bacteriodes and E. coli, which are responsible for diarrhea in formula fed babies. Casein in breast milk has an antimicrobial effect, and represents a mechanism for protection against respiratory infections. It was found to inhibit the attachment of Streptococcus pneumoniae and Haemophilus influenzae to human respiratory tract epithelium (Aniansson et al 1990). Lactoferrin-IgA dependent bacteriostasis is also an important defense against colonization by pathogens of the gut of breast fed babies. Studies have indicated that breast milk contains IgA antibodies to E coli and Vibrio cholerae enterotoxin following previous environmental exposure to these organisms (Holmgren et al 1976). The presence of secretory IgA antibodies directed against these enteric pathogens is believed to result from migration of lymphocytes from the gut associated lymphoid tissue to the mammary gland (Stade et al 1987).

1.6.2 Antiviral Immunity

Evidence has accumulated that breast feeding protects against upper respiratory viral infections and specifically against respiratory syncitial virus infection. Protection against these infections occurs not only by interference of virus adhesion to target cells by IgA, but it is now recognized that the triglyceride content of human milk represents another potentially important anti-infective property. The fatty acids and monoglycerides released when the triglyceride is hydrolysed, either in vitro by lipases of human milk or in vivo by the lipases of the gastrointestinal tract, are potent inactivators of enveloped

viruses as well as of Giardia lamblia , a protozoan causing diarrhea in infants (Isaacs et al 1990). In addition colostral cells play an immunoactive secretory role in antiviral immunity. Colostral cells secrete immunoactive substances such as interferon gamma when stimulated by virus infections. Human milk also has beneficial effects in human immunodeficiecy virus (HIV) infections (Newburg et al 1992). HIV is transmitted through breast milk and a factor in milk inhibits binding of HIV to the CD4 receptor. The factor is found in both seropositive and negative mothers and by blocking a crucial step in virus adhesion and replication may play an important role in HIV infections.

1.6.3 Transfer of Cell Mediated Immunity

Human newborns are generally unresponsive to the antigens that elicit delayed type hypersensitivity skin responses in their mothers (Hayward et al 1983). Mohr et al (1973) have found that the frequency of PPD skin reactivity was much higher in infants who had been breast fed by their PPD positive mothers than in non breast fed controls. Schlesinger et al (1977) have shown the transmission of lymphocyte responses to tuberculin by breast feeding. Bertotto et al (1993) have documented that a significant number of infants born to tuberculin positive mothers have PPD reactive peripheral blood T cells after four weeks of breast feeding, whereas bottle fed offsprings of positive mothers or breast fed sucklings of negative mothers have none. They have also shown that Mycobacteria reactive T cells in breast milk are mainly γ/δ TCR positive cells. These results are suggestive of transfer of cell mediated immunity through milk.

1.6.4 Protection against Urinary Tract Infections in Breast fed Babies

Coppa et al (1990), have suggested that milk may protect against infections of the urinary tract. The neutral oligosaccharides present in human milk caused inhibition of bacterial adhesion to the urinary tract epithelium. Oligosaccharides in milk can also inhibit the adhesion of Streptococcus pneumoniae to human pharyngeal and buccal

epithelial cells (Andersson et al 1986). Colostrum has been shown to contain substantial amounts of oligosaccharides.

1.6.5 Protection from Allergic Reactions

Breast feeding protects vulnerable infants from allergic reactions. The duration of breast feeding resulting in most of the allergy preventing effect was fairly short, from 1 week to 2 months of breast feeding (Juto et al 1982). Studies have shown that exclusively breast fed infants were free of eczema at six months of age, compared with formula fed controls who developed eczema (Mathew et al 1977). All the participants had a positive family history of allergic reactions . Infants of either group had high levels of serum IgE. Protection of breast fed babies was still apparent at 3 years of age in families with allergic histories (Mathew et al 1977, Juto et al 1982, Chandra et al 1979).

The gastrointestinal tract of the newborn has to protect itself from the extrauterine environment by development of a mucosal barrier against the penetration of proteins and protein fragments. During the immediate postpartum period, especially in premature and small-for-date infants, the mucosal defense system is incompletely developed. Therefore susceptibility to infection may result, as well as the potential for hypersensitivity reactions and the formation of immune complexes (Walker et al 1987). Human milk passively protects the vulnerable newborn against the dangers of a deficient intestinal defense system. Much of the protective effects of breast milk is by the presence of secretory IgA antibodies which coat the immature intestinal tract of the infant and prevent antigen penetration.

Melnik et al (1989) have postulated that a relative deficiency in dihomo-gamma-linolenic acid derived prostaglandin E1 is the major etiologic factor for diminished T cell differentiation and function postpartum. Prostaglandin E1 precursors are physiologically provided to the infant by colostrum and milk. Depressed cell mediated immunity and uncontrolled B cell response with increased IgE synthesis seen in atopic individuals correlate with a low supply of prostaglandin E1 precursors during early infancy. These

authors suggest that atopy is a metabolic disorder and breast fed babies are protected from this disorder. Immunomodulatory factors present in colostrum may also have an important influence in the prevention of allergic reactions in the infant.

1.6.6 Breast feeding and Immunizations

Breast feeding, when compared to formula , significantly enhanced T cell mediated response to BCG vaccine given within one month after birth (Pabst et al 1989) and enhanced B cell immunity to polyribose phosphate of Haemophilus influenzae type 'b' conjugate vaccine (Pabst & Spady 1990). Breast fed infants also showed better serum and secretory response to parenteral and peroral vaccines like poliovirus, diphtheria, and tetanus toxoid when compared to formula fed groups (Hahn-Zoric et al 1990). Serum IgG to diphtheria toxoid and serum neutralization of poliovirus at 21 to 40 months of age, saliva secretory IgA to tetanus, diphtheria toxoid and poliovirus at 3 and 4 months of age were higher in the breast fed group. Hahn-Zoric et al , have suggested that the better antibody response in breast fed infants to parenteral as well oral vaccines in serum and secretions could be due to the priming of these infants via antiidiotypic antibodies in breast milk.

1.6.7 Advantages of Breast Milk to Premature Infants

Colostrum with its highly concentrated immunologically active ingredients has an even more important role in the preterm infant than in the full term infant. The major antiinfective factors IgA, IgG, IgM, lactoferrin and lysozyme are significantly higher in preterm colostrum suggesting a physiological role to the preterm infant (Mathur et al 1990). Secretory IgA in milk and colostrum plays an important role in the prevention of necrotizing enterocolitis in the preterm neonate. The mean cell count in preterm colostrum is 9338 cells/mm^3, while in full term colostrum it averaged 5594 cells/mm^3. The absolute cell counts of macrophages, lymphocytes and neutrophils are higher in preterm than in term colostrum. Similarly, total and differential cell counts are

significantly higher in preterm milk compared to full term milk (Jain et al 1991). These high concentrations of immunocompetent cells in preterm milk and colostrum may be a natural protective mechanism to endow the immunocompromised preterm baby with extrinsic immunologic support.

In addition to the immunological benefits stated above breast milk has other beneficial effects on the preterm infant. Lucas et al (1992), have shown that preterm babies who were breast fed developed a higher IQ by 7 1/2 to 8 years than those who did not receive mothers milk. They suggested that breast milk could have a beneficial effect on neurodevelopment. Many possible explanations could be given for this higher IQ in breast fed babies. It could be due to more physical contact or better sense of security. The long chain lipids also play an important role for the structural development of the nervous system. The various hormones and growth factors also influence brain growth and maturation. Preterm infants are particularly sensitive to their early nutrition and preterm breast milk with its immunologic advantages meets their special nutrient needs.

In addition to passive immunity provided by the above mentioned humoral and cellular components , the immunoregulatory factors contained either in milk or elaborated by breast milk cells may also play an important role on the development of the active immune system of the infant. A number of studies have indicated that unfractionated supernatants of milk cell cultures selectively stimulate the production of IgA by peripheral blood cells and cord blood cells (Pittard et al 1979, Puskas et al 1983). It has also been demonstrated that cell free, defatted, filtered human colostrum stimulates murine splenic B lymphocytes to produce immunoglobulins (Juto et al 1985). Crago et al (1981) have documented humoral components in colostrum that inhibit the pokeweed mitogen induced response of normal peripheral blood lymphocytes. Mincheva-Nilsson et al (1991) have shown factors in human colostrum that both stimulate and suppress the immune system. Hooton et al (1991) have documented an activity in human colostrum that inhibits the production of interleukin 2. Breast feeding has also been shown to

reduce the incidence of the autoimmune disease IDDM (Mayer et al 1988) and the chronic inflammatory gut disease described by Crohns (Koletzko et al 1989). Immunosuppressive factors in colostrum might play an important role in the prevention of these conditions. The presence of these factors in colostrum may enable the immature immune system of the neonate to differentiate between self and nonself antigens more effectively than it would otherwise be possible.

1.7 Interleukin 2 and Regulation of its Gene Expression

Interleukin 2 is the principal cytokine responsible for the progression of T lymphocytes from G1 to S phase of the cell cycle. It is produced by CD4+ T cells and in lesser quantities by CD8+ T cells and serves as both an autocrine and paracrine growth factor for T cells. Normally IL2 is transcribed, synthesized, and secreted by T cells only upon activation by antigens or by treatment with pharmacological agents which mimic antigenic stimulation. Synthesis of IL2 is restricted to T cells, and like other members of the lymphokine gene, family transcription of the IL2 gene is rapidly and transiently activated upon T cell stimulation. IL2 mRNA is detectable within 1 to 6 hours of stimulation and has a half life of 1 hour or less (Shaw et al 1987). The level of IL2 mRNA depends on mechanisms like frequency of transcription initiation, rate of transcription and the stability of mRNA.

Transcriptional activity of the IL2 gene is determined by nuclear factors that interact with specific elements in the regulatory region of the gene (Crabtree et al 1990). The IL2 gene is controlled by a 267 base pair enhancer extending from -52 to -326 nucleotides 5' to the transcription initiation site of the gene (Fujita et al 1986). This enhancer region contains all the information for T cell specific inducible activation of IL2 transcription. A highly cooperative interaction between various nuclear factors has to occur, each of which follows a different activation schedule to allow activity of the enhancer of the IL2 gene. The enhancer region contains a combination of different

protein binding motifs to which functionally relevant nuclear proteins bind , namely nuclear factor of activated T cells (NF-AT) binding site, NF-kB like motif, two AP-1 like binding sites, and a site for NF-IL2 A. All of these protein binding sites contribute to the activity of the IL2 enhancer. Signals that activate only one of the enhancer elements will not give rise to the IL2 gene transcription. Transcription factors involved in the regulation of the IL2 gene are constitutively expressed proteins such as Oct-1 and AP-3, and inducible binding proteins such as NF-AT, NF-kB and AP-1. NF-AT appears to be a critically important transcriptional factor for IL2 gene expression (Shaw et al 1988b). It binds the sequence between -264 to -284 of the enhancer region in the human IL2 gene. NF-AT is made up of two components and the formation of a functional NF-AT complex requires new protein synthesis. The preexisting cytoplasmic subunit of NF-AT translocates to the nucleus in response to signalling from the antigen receptor and it combines with the newly synthesized nuclear subunit to mediate transcriptional enhancement of the IL2 promoter. Known immunosuppressants like Cyclosporine-A and FK506 inhibit the synthesis of IL2. They specifically block the calcium arm of the T cell activation signal. These drugs block the calcium dependent nuclear translocation of the cytoplasmic subunit of NF-AT , thereby inhibiting the binding of NF-AT to the IL2 enhancer and transcriptional activation mediated by it.

1.8 Objectives Of The Study

The general objective of the research is to study immunosuppressant factors in colostrum. The specific aims of the study are as follows.

1. To study the effect of colostrum on the production of interleukin 2 in various T helper cell lines,
2. To determine the mode of action of colostrum inhibitory factor, by studying the effect of the inhibitory factor on the induction of IL2 mRNA,

3. To determine the approximate molecular weight of colostrum inhibitory factor and the effect of acidification on the inhibitory activity,
4. To study whether the colostrum inhibitory factor is different from any of the known TGFβ group of immunosuppressants, and
5. To test if bovine colostrum has a similar activity and, if so, to use bovine colostrum for further characterization of the activity.

2. MATERIALS AND METHODS

2.1 Reagents

Concanavalin A (Con A) , phorbol 12-myristate 13-acetate (PMA), and ionomycin were obtained from Sigma Chemical Company (St Louis, MO). Stock solutions of PMA and ionomycin were made up in dimethylsulfoxide (DMSO). Recombinant human IL2 was secreted from yeast cells transformed with an expression vector containing a synthetic human IL2 cDNA (Barr et al 1984). Human recombinant transforming growth factors β (TGFβ1 and -β2) and neutralizing rabbit antibody to TGFβ1, -β2 and panspecific antibody to TGF β were obtained from R&D Systems (Minneapolis, MN). Cyclosporine A (CsA) was a gift from Sandoz Canada Inc., Dorval, Quebec. Radionucleotide, [α-^{32}P] deoxycytidine 5'-triphosphate, tetra-(triethylammonium) salt was obtained from New England Nuclear, Lachine, Quebec. Molecular biological reagents and enzymes were obtained from Gibco BRL, Life Technologies, Burlington, Ontario. Anti-CD3 antibody was obtained from Dr. Bleackly's lab.

2.2 Cell Lines and Culture Conditions

Cell lines were maintained in RHFM (RPMI, Hepes, fetal bovine serum, and mercaptoethanol) consisting of RPMI 1640 (Gibco BRL) supplemented with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.4], 100 μM 2-mercaptoethanol, 100 μg/ml streptomycin, 100 IU/ml penicillin and 10% heat inactivated fetal bovine serum (FBS; Hyclone). MTL 2.8.2 (mouse cytotoxic T cells) IL2 dependent cell lines were maintained in RHFM containing 30 U/ml human recombinant IL2. Cell lines used in the study were: EL4.E1 (C57B1/6 mouse T lymphoma, Farrar et al 1980), Jurkat (human T leukemia), MTL 2.8.2 (mouse CTL, Bleackley 1982) and 12.1.19 (T-

cell hybridoma, Fotedar 1985). Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers and were prepared by centrifugation of blood over Ficoll-Paque (Pharmacia, Uppsala, Sweden). For these experiments 10% AB serum was substituted for fetal bovine serum. Cells were grown in 75 cm^2 tissue culture flasks (Corning, Corning N.Y.) at 37 ^0C with 5% CO_2 in a humidified incubator.

EL4.E1 cells secrete IL2 in response to stimulation by PMA (10 ng/ml) alone, or IL2 production can be enhanced by costimulation with the calcium ionophores, ionomycin or A23187. The human T cell line Jurkat requires two stimuli to maximally induce IL2 production; PMA (20 ng/ml) was used in combination with ionomycin (2μM). The T-cell hybridoma 12.1.19 produces IL2 upon stimulation with anti-CD3 antibodies alone. Anti-CD3 antibody was diluted 1000 times using Tris pH 9.0 at a final concentration of 10 mM. 200μl of the diluted antibody was added to a 96 well flat bottomed plate and incubated at 37^o C for one hour. The wells with antibody were washed thrice with RHFM medium. 12.1.19 cells at a concentration of $2x10^6$ /ml were added to the wells. The positive controls are wells coated with antibody to which cells were added and the negative controls are cells alone, added to wells without antibodies.

2.3 Colostrum

Colostrum samples were collected within one day of birth from healthy women who delivered their infants at term at University of Alberta Hospitals. Samples were centrifuged (1500g, 15 min, room temperature). The supernatant fluid was retained (Fraction 2, F2), avoiding pelleted cells and the overlying fat pellicle. After testing samples were pooled (groups of six), and stored at -70°C.

F2 was acidified by adding 5N HCl to pH 3.0, held for five minutes at room temperature, and then neutralized with 5N NaOH/1M HEPES, to generate F2A. Bovine colostrum was obtained from the University of Alberta farm and was treated in the same way as human colostrum.

Colostral proteins were separated into whey and casein fractions by acidification and calcium precipitation (Kunz et al 1989). To 40 ml of defatted, cell free colostrum 3-4 drops of 6M HCl was added to lower the pH to 4.3 and left on ice for one hour. 2ml of 1.2M calcium chloride (final concentration of 20mM) was added to the above and left on ice for a further 10 minutes. The above treated colostrum was then centrifuged at 150,000 x g for 1 hour in a Ti 75 rotor (Beckman ultracentrifuge) at 20^0 C, resulting in separation into a clear supernatant (whey), a precipitate (casein) and a fat layer on top. The casein fraction was reconstituted to the original volume by water and both the whey and dissolved casein fractions were dialysed against PBS pH 7.4 overnight.

2.4 Proliferation Assays

PBMC (10^5) were cultured in triplicate for 24 hours in a 0.2 ml final volume in 96-well flat-bottomed microtitre plates. 0.25 mCi/well [^{3}H]-TdR (2 Ci/mM, NEN, Boston, MA) was added for the final 6 hours. Cultures were harvested on a Skatron harvester (Lier, Norway) and the radioactivity incorporated was determined by β-scintillation counting.

2.5 Mixed Lymphocyte Reaction (MLR)

Peripheral blood mononuclear cells were obtained from two healthy volunteers. 10^5 cells were irradiated at 1500 rad (^{137}Cs source) to be used as stimulator cells . 10^5 responder cells were mixed with 10^5 stimulator cells in a 96-well flat-bottomed microtitre plate and incubated for 5 days. [^{3}H -TdR] was added for the final 6 hours and the radioactivity incorporated was determined by β scintillation.

2.6 Assay for IL2

Colostrum at a final concentration of 10% was added to $2x10^6$/ml stimulated EL4.E1 or Jurkat cells at the start of culture in a 0.2 ml final volume in a 96-well flat-bottomed microtitre plate (Costar). The plate was incubated for 24 hours at 37^0 C and 5% CO_2. Supernatant media were collected from the activated cell cultures and tested for IL2 as described previously (Hooton et al 1985) using the IL2 dependent murine cell line MTL 2.8.2 and monitoring the reduction of the tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Mosmann, 1983). IL2 activity was expressed relative to a reference standard from Biological Resources, NCI-FCRF (Frederick,MD).

2.7 Effects of anti-TGFβ antibody

Graded concentrations of anti TGFβ (50 µg/ml to 10 µg/ml) antibody were added to the wells at the start of culture. The plate was incubated for 24 hours and the supernatant medium was tested for IL2.

2.8 Gel Exclusion Chromatography of Colostrum

Defatted, cell free colostrum was chromatographed on Sephacryl S-300 gel (Pharmacia LKB Biotechnology, Piscataway, NJ) filtration column of 100 ml volume at 4° C. Molecular weight markers were blue dextran (Mr, 2000 kD), BSA (Mr, 68 kD), and thymidine phosphate (Mr, 500 D). Five ml of colostrum was applied to the column. Proteins were eluted from the column with HEPES buffer containing 25mM HEPES and 0.9% Sodium chloride salt. The resultant fractions were tested for the inhibition of IL2 production in stimulated EL4 cells.

2.9 Sucrose Gradient Centrifugation

This technique was used to isolate fat globules from colostrum (Patton et al 1985). Five ml of colostrum was placed in a 12 ml round-bottom tube, and 0.25 g of sucrose was

added. The tube was gently swirled to dissolve the sucrose. A 9-inch Pasteur- type pipette with along capillary was placed in a 15 ml conical tube that had been filled with 10 ml of warm (37^0 C)PBS. The sucrose treated milk was carefully inserted into the open end of the pipette by means of a 6 inch Pasteur pipette fitted with a rubber bulb, avoiding air bubbles. Once all the milk had been transferred the pipette was lifted until its tip reached the interface between the buffer and milk . The pipette was left in this position for some time and then removed from the tube in one smooth motion. The tube and contents were then centrifuged at 1500 x g for 20 minutes at room temperature in a swinging bucket rotor. Colostrum was separated into 4 layers by this technique. Layer 1 contained the fat. Layer 2 was PBS mixed with colostrum. Layer 3 was at the interface . Layer 4 was at the bottom containing most of the protein.

2.10 Protein Determination

All protein determinations were done using the Bio-Rad protein assay (Bio-Rad Laboratories Canada, Mississauga, Ontario) with BSA as a standard.

2 .11 RNA Isolation

Total cellular RNA from 2.5 x 10^7 PMA stimulated EL4 cells treated with 10% colostrum or 300 ng/ml CsA was isolated by a modification of the guanidine thiocyanate-cesium chloride gradient method (Chirgwin 1979). The RNA isolation reagent was prepared by gently heating guanidine thiocyanate (final concentration 4 M), sodium N-lauroylsarcosine (final concentration 0.5%) and sodium citrate (final concentration 2.5 mM). The pH of the solution was adjusted to 7.0 and filtered to remove particulate matter. On the day of use, 2-mercaptoethanol was added to achieve a final concentration of 0.1 M.

The cells were washed twice with PBS and pelleted. The pellet was resuspended in 6 ml of guanidine thiocyanate solution and homogenized by aspiration 5 to 6 times through a 21G needle attached to a 5 ml disposable syringe. The RNA sample was

layered over 4 ml of cesium chloride (5.7 M CsCl, 0.01M EDTA, pH 7.0) and centrifuged for up to 18 hours using a Beckman SW50.1 rotor at 36,000 rpm. The RNA pellet was resuspended in Tris-EDTA buffer (TE,pH 8.0: 10 mM Tris-HCl, 1 mM EDTA) and precipitated by addition of a one tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of ethanol. The precipitate was washed twice in 70% ethanol, resuspended in TE, and quantitated using ethidium bromide fluorimetry.

2.12 Northern Analysis

Approximately 5µg of RNA was denatured for 15 minutes at 55 ^{0}C in a solution containing 50% formamide, 6.5% formaldehyde and 1x MOPS buffer (20mM MOPS, 5mM sodium acetate, 1mM EDTA, pH 7.0). The RNA was size fractionated for 4 hours at 120 V through a 1% agarose gel containing 0.67% formaldehyde and 0.33 µg/ml ethidium bromide in a BRL electrophoresis gel box modified to allow circulation of the MOPS running buffer. RNA was transferred by capillary action onto nylon membrane (Hybond-N, Amersham). The gel was placed face down on a clean glass plate followed by the nylon membrane and two sheets of Whatman 3MM paper presoaked in 10 x SSC (1 x SSC, pH 7.0: 150 mM NaCl, 15 mM sodium citrate). The pile was completed by adding 2 sheets of dry filter paper, two inches of paper towel (all components cut to the size of the gel), a glass plate and a 1kg weight. The structure was left overnight and the RNA was crosslinked to the support using a UV Stratalinker^{TM} for the recommended exposure.

Filters were prehybridized for 8 to 16 hours in solutions containing 50% formamide, 5 x SSC, 5 x Denhardt's solution (100x Denhardt's: 1% Pharmacia Type 400 Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 0.1% SDS, and 200µg/ml denatured, sheared salmon sperm DNA. Hybridizations were carried out in the same buffer for 12 to 18 hours at 40^0 C. The resultant blots were washed 4 times for 15 minutes at 42^0 C and

55°C in 2x SSC and 0.1% SDS and finally in 0.2% SSC, 0.1% SDS at 55° C for 15 minutes. Radiolabelled bands were visualized by autoradiography.

Radioactive Labelling of Mouse IL2 cDNA:

Mouse IL2 cDNA was labelled using an adapted random primer method. The 5x random primer solution was made up by combining 100 parts A (A: 0.5 mM each of dATP, dTTP and dGTP in 1.25 M TRIS-HCl, 0.125 M $MgCl_2$, pH 8.0, 0.25 M 2-mercaptoethanol), 250 parts B (B: 2 M HEPES, pH 6.6) and 150 parts C (C: hexadeoxyribonucleotides [pd(N)6 obtained from Pharmacia, product no. 27-2166, Lot no. 97899] at 90 OD U/ml in TE). For labelling reaction up to 1 µg of DNA in TE was heated for 5 minutes at 95° C, cooled on ice and the following reagents added to achieve a final volume of 50 µl : 10 µl 5x random primer solution, 20 mg BSA, 200 µCi α^{32}P-dCTP (specific activity, 800 Ci/mmol) and 3U Klenow fragment of DNA (GIBCO BRL). The reaction was incubated at 37° C for 3 hours. Unincorporated label was removed using spin columns. For this procedure, a disposable 1 ml syringe barrel was plugged with a small amount of siliconized glass wool and filled with Sephadex G-50 equilibrated in TE, pH 8.0. The bed resin was washed with a further 2 ml of TE. A microcentrifuge tube with the cap removed was placed within a 15 ml culture tube and the syringe column was inserted such that the tip rested within the microfuge tube. The column was spun for 2 min at 1200 rpm and the sample was loaded onto the top of the column in a 50 µl volume. The column was respun under the same conditions and the labelled oligonucleotide was used without further purification.

2.13 Reverse Transcriptase-Polymerase Chain Reaction

2 x10^6 EL4 or Jurkat cells were stimulated with 10 ng/ml PMA or 20 ng/ml PMA and 2µM ionomycin for 18 or 6 hours respectively, in the presence or absence of colostrum (10%) or recombinant TGFβ (1ng/ml). 3x10^4 of the above treated cells were

washed twice in PBS and the pellet was resuspended in 18 µl of buffer solution containing 50mM TRIS-HCl, 75 mM KCl, 3mM $MgCl_2$ (1xBRL buffer, BRL,GIBCO), autoclaved water and RNasin 18U (Promega, Madison, WI). 2µl of 5% NP40 (Nonidet 40) was added to lyse the cells. Samples were heated to 65^0 C for 1 minute, cooled at 22^0 C for 3 minutes, and returned to ice. Cytoplasmic RNA thus obtained was reverse transcribed with Superscript reverse transcriptase, 50 U/assay. The reaction mixture contained 1xBRL buffer, 200 µM of each dNTP, 20mM DTT and 10 U RNasin. The reagents were incubated at 37^0 C for 1 hour, heated to 95^0 C for 5 minutes to denature the reverse transcriptase, cooled on ice and stored at -20^0 C. From each cDNA preparation 2 µl were diluted to a final volume of 20 µl in 1 x Taq Buffer containing 50 mM KCl and 20 mM Tris-HCl pH 8.4, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5ng/µl of sense and antisense IL2 specific primers and 5 units Taq polymerase. The samples were heated at 94^0 for 3 minutes and then subjected to 25 PCR cycles. Each cycle consisted of denaturing at 94^0 for 1 minute, annealing at 55^0 for 1 minute and primer extension at 72^0 for 1.5 minutes. At the end of 25 cycles samples were subjected to final extension at 72^0 C for 10 minutes. Following amplification, 10 µl of the reaction product was mixed with 2 µl of stop solution (60% glycerol, 1% SDS, 50 mM EDTA and bromphenol blue) and 10 µl was applied to a 2% agarose gel in TBE buffer. The gel loaded with the samples was electrophoresed at 125 V for [illegible]ars. Molecular weight standard included 10µl of a 1kb PM2-Hae III DNA (Boehringer, Mannheim, FRG). The DNA bands on the gel were visualized by ethidium bromide fluorescence. In all experiments the presence of contaminants was checked by control reactions in which amplification was carried out on samples in which lysis buffer alone was added to the reverse transcription reaction mixture and PCR buffers alone added to Taq polymerase.

3 RESULTS

In earlier experiments from this lab done by Dr. Hooton (1991), F2 human colostrum inhibited lectin- and alloantigen- induced T lymphocyte proliferation but did not inhibit the proliferation of autonomously growing cells. Results of some of these experiments are presented in Sections 3.1 and 3.2. The fact that colostrum inhibited the proliferation of T cells in immune or immunomimetic responses, but did not inhibit the proliferation of autonomously growing cells, suggested that an intermediate process in the immune response was being inhibited. An obvious candidate was the production of IL2, a lymphokine necessary for the growth of T cells. Hooton et al (1991) documented that the inhibition of the proliferation of antigen stimulated T cells was due to the presence of a factor in F2 human colostrum which inhibited the production of IL2. The balance of the results presented in this thesis are directed toward characterizing this Colostrum Inhibitory Factor (CIF).

3.1 Inhibition of lectin and alloantigen induced T- lymphocyte proliferation by colostrum fraction F2.

PBMC from adult human volunteers were incubated with Con A (3µg/ml) or PHA (4µg/ml) in the presence or absence of 10% colostrum for 3 days. Both lectins stimulate T cell proliferation and in both cases colostrum inhibited this proliferation (Fig.3.1). A similar inhibition of proliferation is observed with one and two way MLRs set up with human PBMC as shown in Figure 3.2 (Hooton et al 1991).

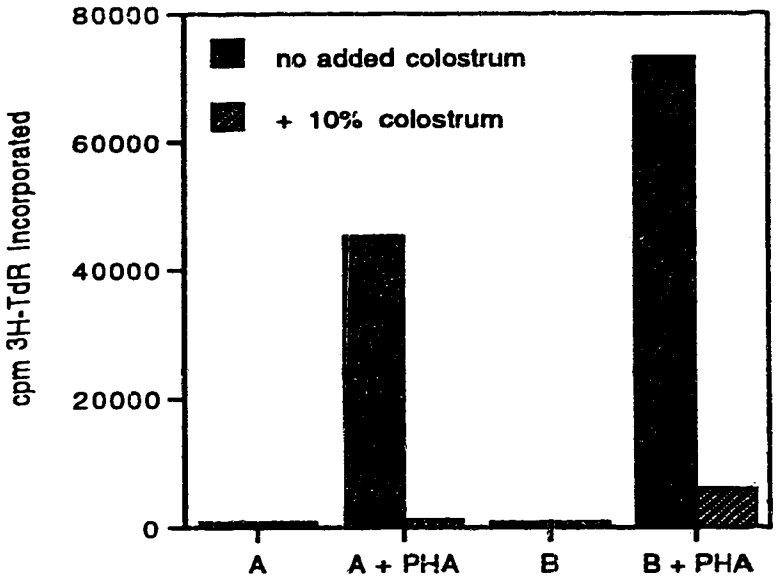

Figure 3.1: Effect of human colostrum on the proliferation of PHA stimulated PBMC. 10^5 PBMC from two healthy adult volunteers (A or B) were incubated for three days in the presence of medium only, or 4 μg/ml PHA, with or without 10% F2 human colostrum. Proliferation was measured by [^{3}H]TdR incorporation (Taken from Hooton etal).

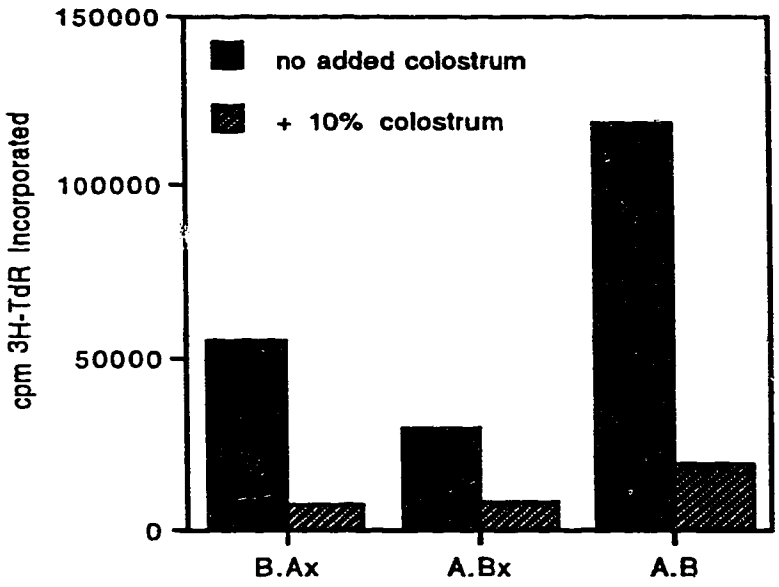

Figure 3.2 : Effect of human colostrum on one and two-way MLRs. 10^5 PBMC from healthy adult volunteers (A or B) were incubated in the presence or absence of 10% colostrum fraction 2 for five days. Ax and Bx refer to irradiated stimulator cells (Taken from Hooton et al).

3.2. Inhibition of IL2 production in various stimulated T helper cell lines by F2.

Fraction F2 colostrum inhibited the production of IL2 in EL4 cells stimulated with PMA alone (Fig. 3.3) or with PMA and ionomycin (Fig. 3.4), in Jurkat cells stimulated with either PMA and Con A (Fig. 3.5) or PMA and ionomycin (Fig. 3.6). F2 also inhibited IL2 production in the murine T hybridoma cell line 12.1.19 stimulated with anti-CD3 (Fig. 3.7). This effect was apparent with all colostrum samples tested as shown in Figure 3.8 (Hooton 1991). Inhibition was apparent with as low a concentration as 1% colostrum.

To determine whether the inhibition of IL2 production by human colostrum could be explained by colostrum being toxic to cell lines, the proliferation of two transformed cell lines was investigated in the presence of varying concentations of colostrum. Colostrum added to either a human T cell (Jurkat) or a human B cell (RPMI 8666) tumor cell line had no effect on proliferation as measured by ^{3}H- thymidine incorporation and is shown in Figure 3.9 (Hooton 1991). Colostrum at 10% was also not toxic to any of the T helper cell lines used, as determined by vital dye staining (eosin) at the end of culture.

To determine whether F2 human colostrum might have exerted its effect by inhibiting the response of MTL indicator cells to IL2, 10% colostrum was added to MTL cells and incubated with varying concentrations of recombinant human IL2 in a 24 hour assay, proliferation being assesed by monitoring the reduction of the tetrazolium dye MTT. As shown in Fig. 3.10 colostrum had no significant effect on the IL2 dependent proliferation of MTL cells. Moreover, neither PMA nor ionomycin at the concentrations used to stimulate cells had any effect on the proliferation of MTL cells.

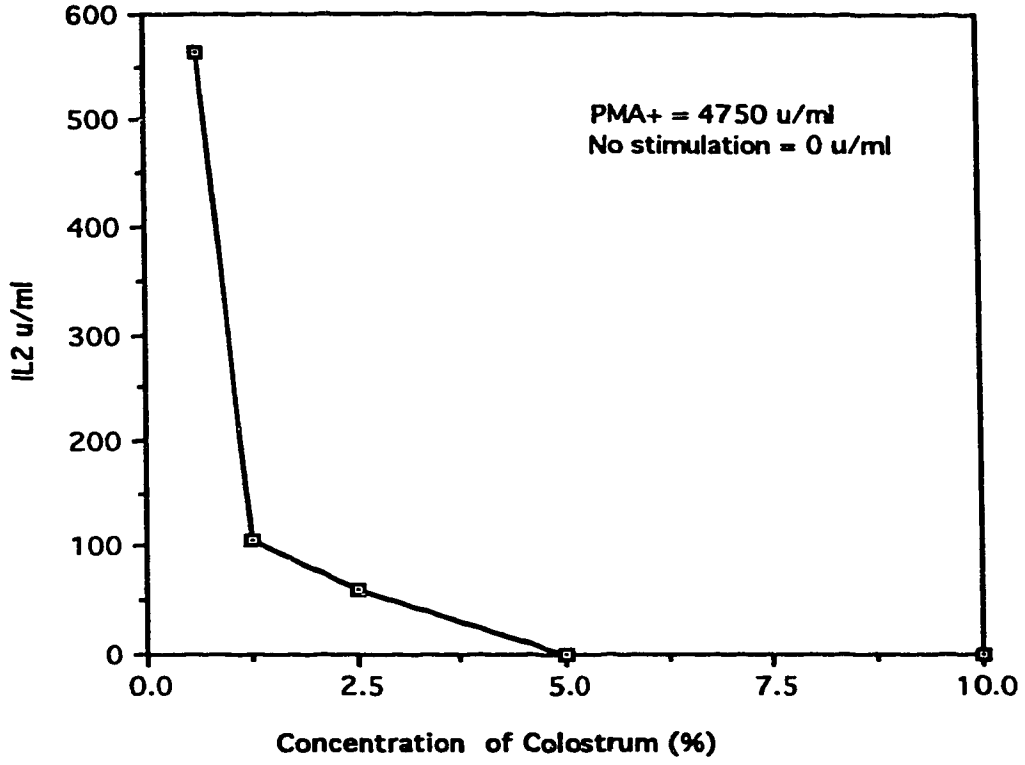

Figure 3.3 : Effect of F2 human colostrum on IL2 production in PMA-stimulated EL4 cells. 2 x 10^6 /ml EL4 cells were incubated for 24 hours with 10 ng/ml PMA and graded concentrations of pooled F2 human colostrum in a final volume of 0.2 ml RHFM. 100 µl supernatant medium was removed and assayed for IL2 in the bioassay using MTL2.8.2 cells.

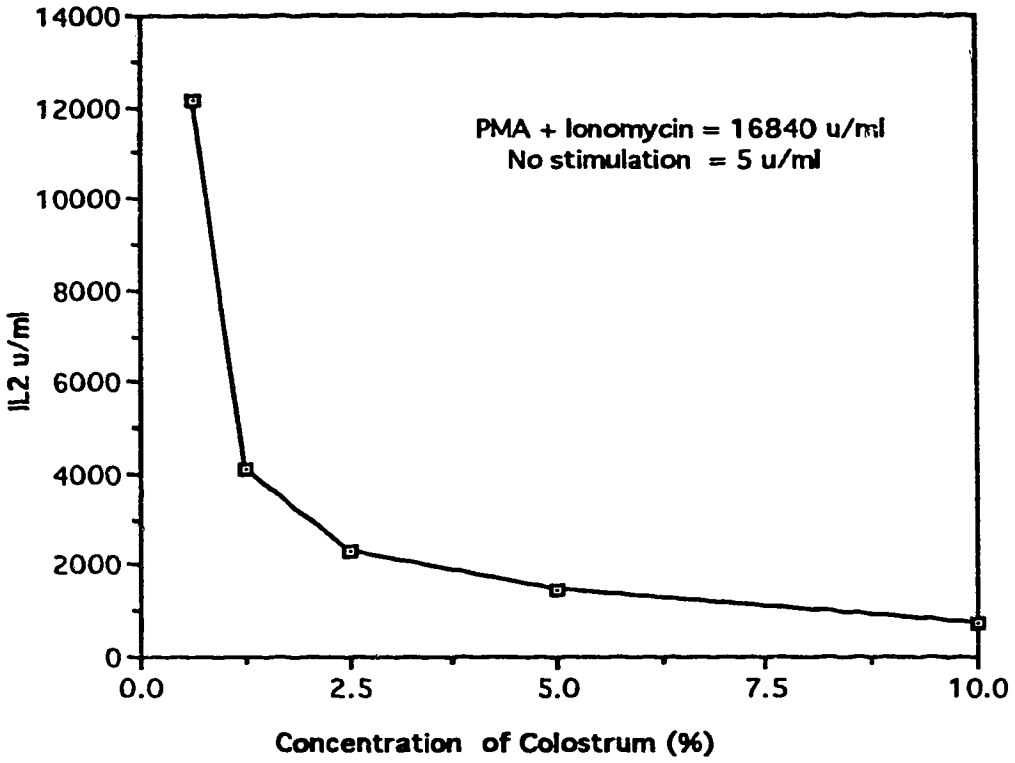

Figure 3.4 : Effect of F2 human colostrum on IL2 production in EL4 cells stimulated with PMA and ionomycin. 2 x 10^6 /ml EL4 cells were incubated for 24 hours with 10 ng/ml PMA, 2 µM ionomycin and graded concentrations of pooled F2 human colostrum in a final volume of 0.2 ml RHFM. 100 µl supernatant medium was removed and assayed for IL2 in the bioassay using MTL2.8.2 cells.

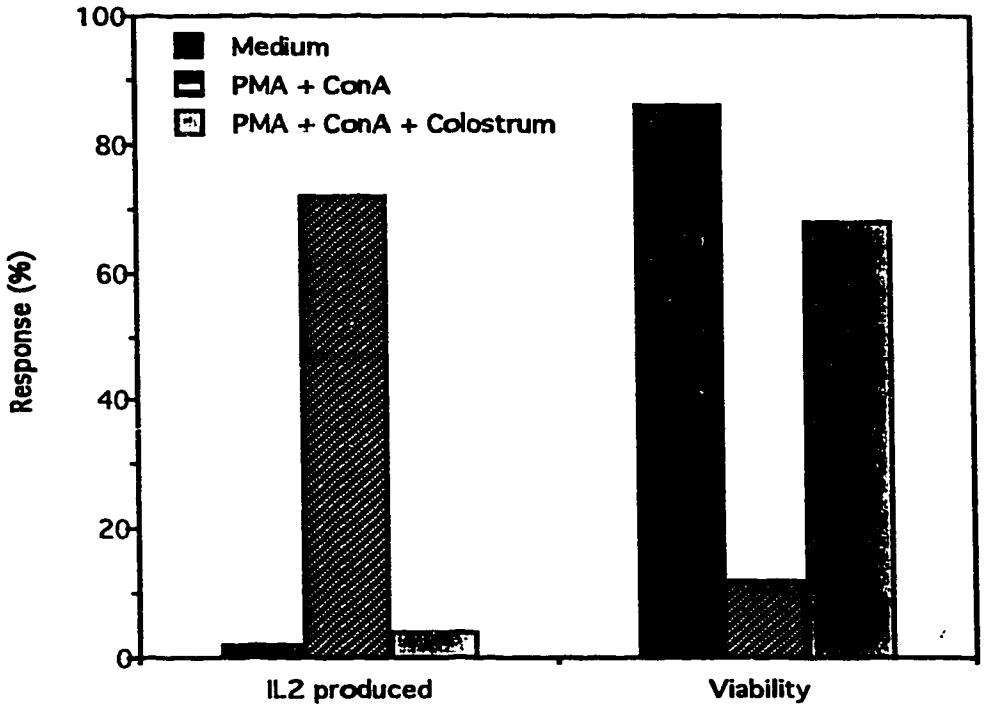

Figure 3.5 : Effect of colostrum on the viability of, and production of IL2 in Jurkat cells stimulated with PMA (10 ng/ml) and ConA (30 μg/ml) with and without 10% F2 colostrum (Hooton et al 1991).

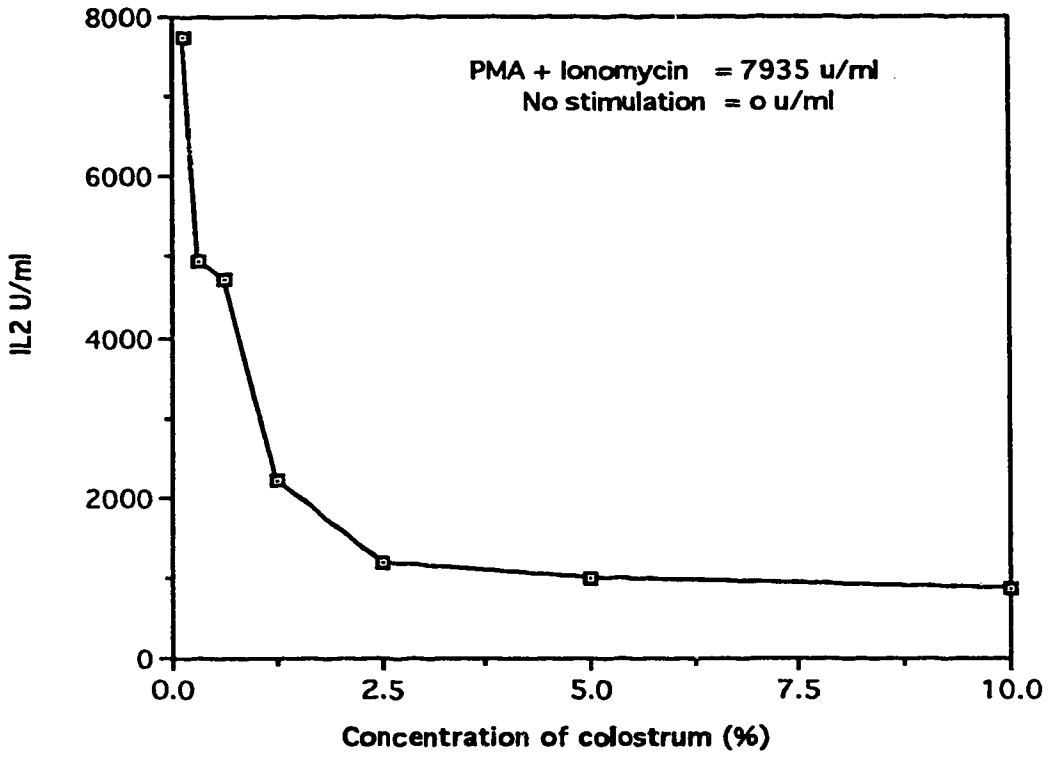

Figure 3.6: Effect of F2 human colostrum on IL2 production in Jurkat cells stimulated with PMA and ionomycin. 2 x 10^6 /ml Jurkat cells were incubated for 24 hours with 20 ng/ml PMA, 2 µM ionomycin and graded concentrations of pooled F2 human colostrum in a final volume of 0.2 ml RHFM. 100 µl supernatant medium was removed and assayed for IL2 in the bioassay using MTL2.8.2 cells.

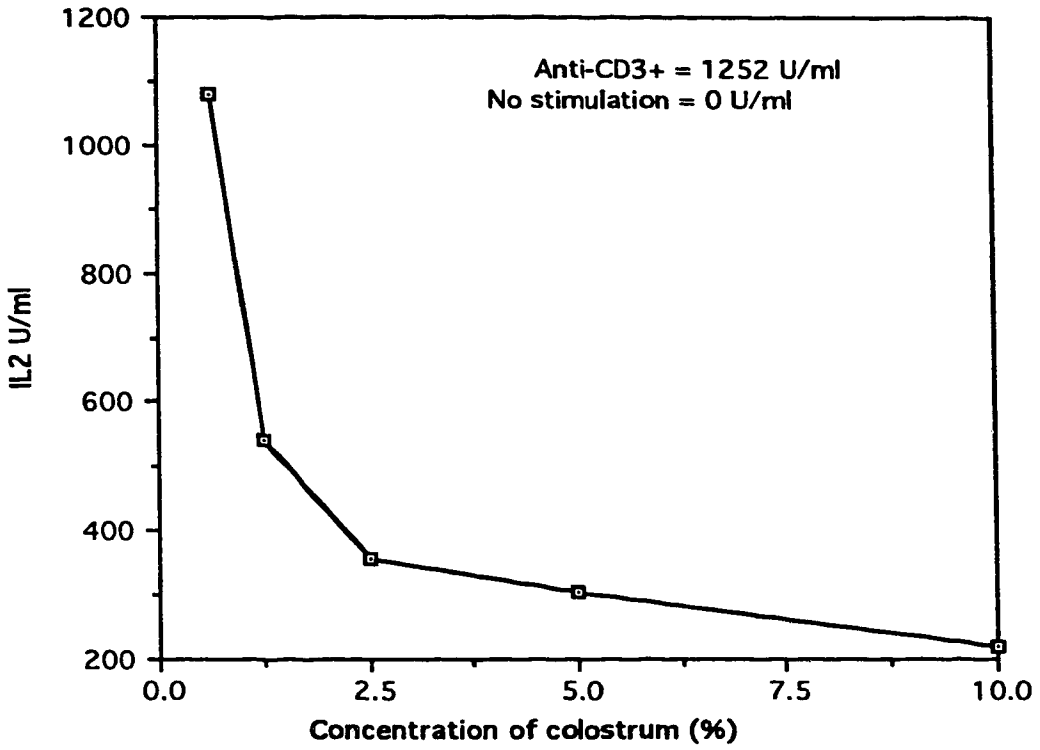

Figure 3.7: Effect of F2 human colostrum on IL2 production in T-hybridoma cell line 12.1.19 stimulated with anti-CD3. 2 x 10^6 /ml 12.1.19 cells were incubated with various concentrations of F2 human colostrum in a 96 well plate coated with anti-CD3 antibody. Supernatants were harvested after 24 hours and assayed for IL2 using MTL2.8.2 cells.

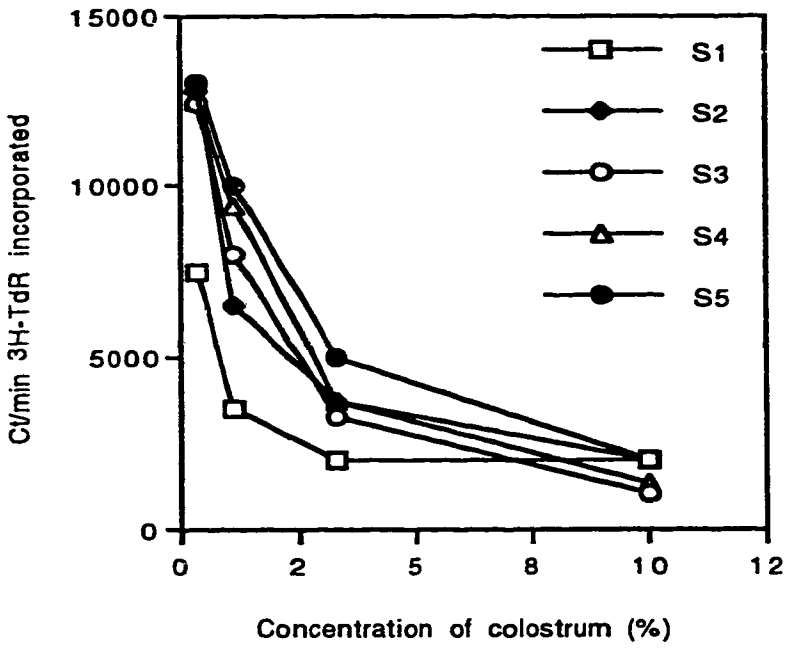

Figure 3.8: Effect of F2 human colostrum from individual donors on IL2 production in Con A stimulated PBMC. S1 to S5 represent colostrum samples from individual donors (Taken from Hooten et al).

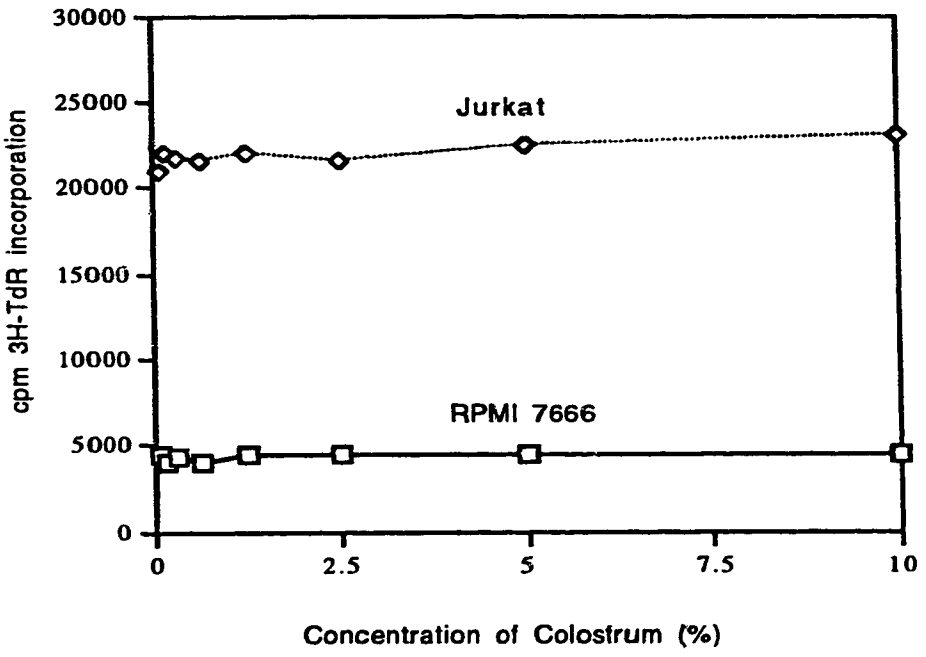

Figure 3.9: Effect of F2 human colostrum on the proliferation of human lymphocyte tumor cell lines: Cells were incubated with graded doses of F2 colostrum and proliferation was measured by [^{3}H]-TdR incorporation (Taken from Hooton et al 1991).

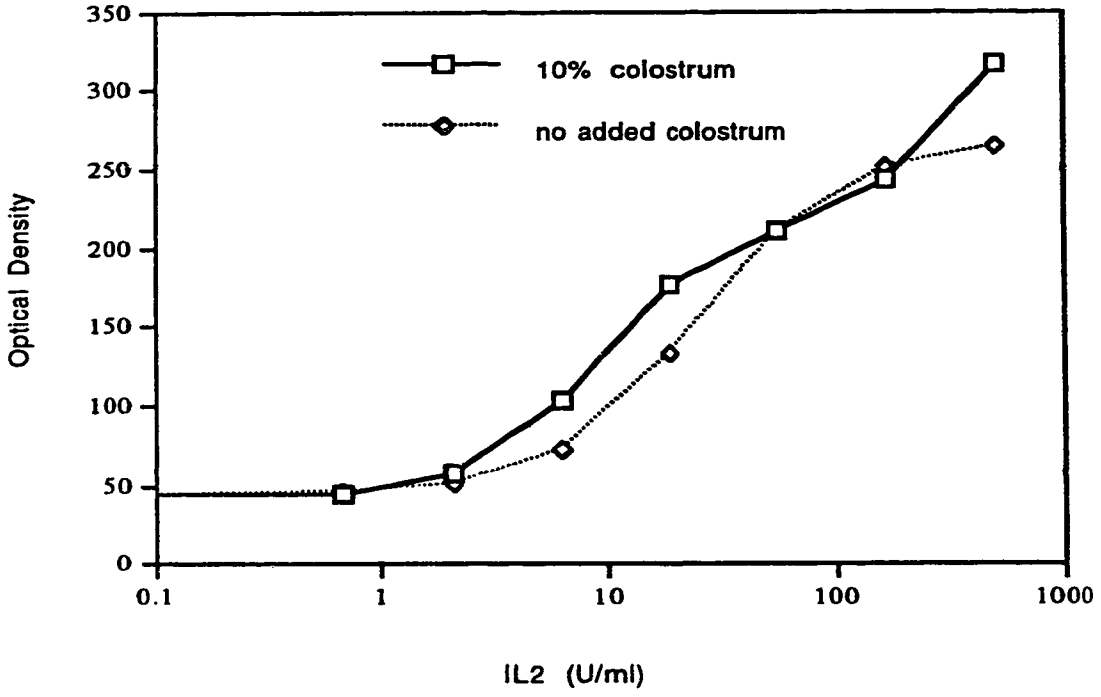

Figure 3.10: Effect of F2 human colostrum on the IL2 - dependent proliferation of MTL 2.8.2 cells. 10^4 MTL cells per well were incubated with graded doses of recombinant IL2 in the presence and absence of 10% F2 colostrum in a final volume of 0.2 ml RHFM. Cells were incubated for 48 hours and the response was determined by the MTT dye reduction method.

Preliminary studies have shown the gradual disappearance of the colostrum inhibitory factor from breast milk by 35 to 50 days postpartum (Fig. 3.11). In contrast to the lack of toxicity seen in assays to which colostrum was added, human milk taken between 2 and 5 weeks was toxic to both stimulated and unstimulated Jurkat and EL4 cells. The toxicity was not specific to a single donor. This toxicity was not due to complement mediated lysis as milk samples heated to 56^0 C for 30 minutes (which inactivates complement) were still toxic.

3.3. Fraction 2 colostrum inhibitory factor (CIF) inhibits the accumulation of IL2 mRNA in PMA stimulated EL4 cells:

EL4 cells were stimulated with 10 ng/ml PMA to induce the accumulation of IL2 mRNA, and either F2 human colostrum or cyclosporine A (CsA), were added at the outset of the cultures. Crude mRNA was extracted from the cells after 24 hours and subjected to Northern analysis using mouse IL2 cDNA probe (Fig. 3.12). Control cells contained no detectable IL2 mRNA, and stimulation with PMA induced its accumulation. CsA at 300 ng/ml completely suppressed this response, as shown earlier (Shaw et al 1987). F2 human colostrum at 10% v/v also suppressed the accumulation of IL2 mRNA. These results were confirmed using reverse transcripatase - PCR analysis (Fig. 3.13). They indicate that F2 colostrum, like CsA acts at the level of mRNA accumulation, although they do not determine whether the effect is on transcription rate per se, as it is with CsA. Recombinant human TGFβ1 at 1 ng/ml inhibits induction of IL2 mRNA to a similar degree as F2 CIF, as detected by RT-PCR (Fig.3.13).

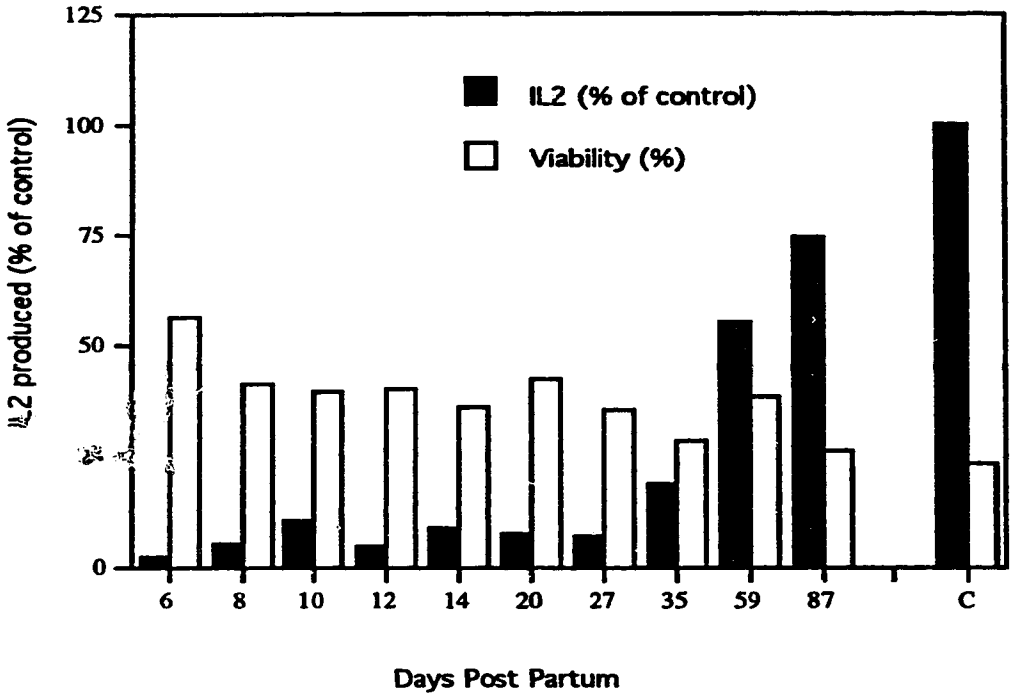

Figure 3.11: Effect of serial milk samples on the viability and IL2 production in Jurkat cells stimulated with PMA and Con A. C represents stimulated cells without colostrum.

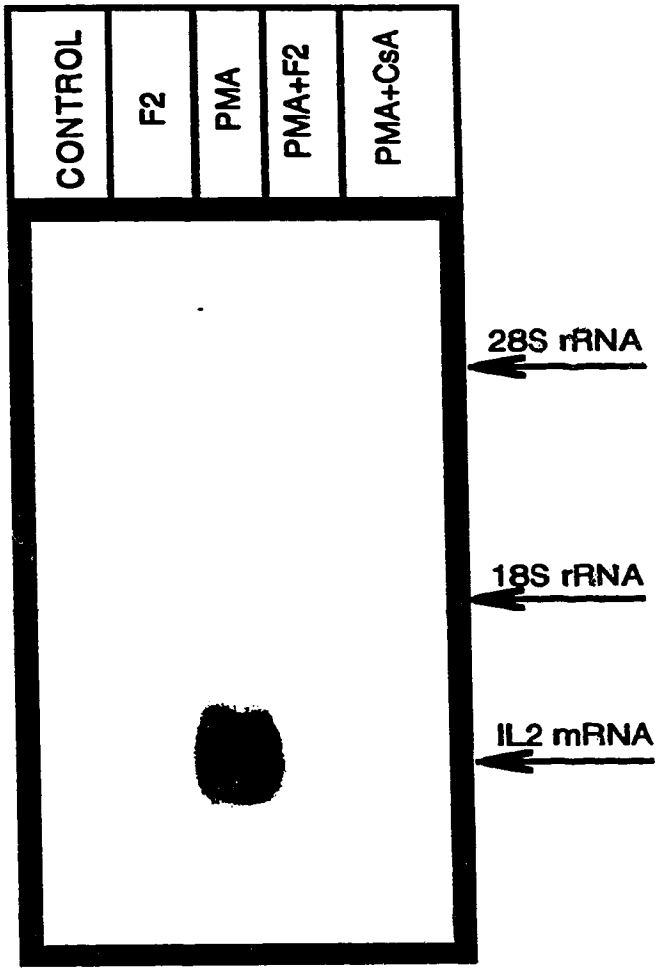

Figure 3.12: Effect of human colostrum on induction of IL2 mRNA in PMA stimulated EL4 cells, as detected by Northern blot analysis. EL4 cells were stimulated with PMA, and RNA was extracted after 24 hours and examined by Northern blot. The first lane contains RNA from unstimulated cells. The second, RNA from cells exposed only to 10% v/v F2 human CIF. The third lane shows a single band due to IL2 mRNA induced by 10 ng/ml PMA. The fourth lane shows the inhibitory effect of having 10% v/v F2 CIF present during stimulation with PMA. The last lane shows the inhibitory effect of 300 ng/ml CsA on PMA-induced IL2 mRNA accumulation.

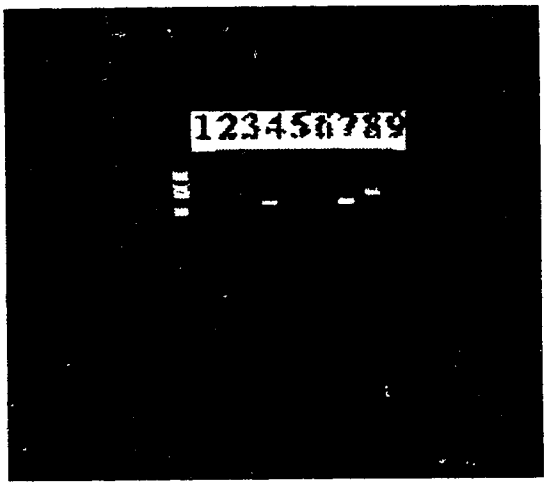

Figure 3.13: Effect of TGFβ and colostrum on IL2 mRNA induction in EL4 cells as detected by Reverse Transcriptase - Polymerase chain reaction. EL4 cells were stimulated with PMA, and RNA was extracted after 18 hours by lysis with NP40 (a detergent). The RNA was examined by RT-PCR.

DNA marker: PM2 DNA cut with Hae III
Lane 1: Stimulated cells + defatted cell free colostrum (F2, 10%)
Lane 2: Stimulated cells + acidified F2 (F2A, 10%)
Lane 3: Stimulated cells + TGFβ (1ng/ml)
Lane 4: Stimulated cells (PMA 10 ng/ml)
Lane 5: Unstimulated cells
Lane 6: RT negative control (buffers alone added to reverse transcriptase reaction)
Lane 7: mouse IL2 mRNA (1pg/μl) - RT positive control
Lane 8: pGEM1 HIND III IL2 DNA - PCR positive control
Lane 9: PCR negative control (PCR buffers alone added to Taq Polymerase)

3.4. Sephacryl S-300 Gel exclusion chromatography of Fraction F2 colostrum.

F2 colostrum was subjected to gel exclusion chromatography on sephacryl S-300 with a fractionation range between 1 x 10^4 to 1.5 x 10^6. Proteins were eluted from the column using HEPES and NaCl buffer. The resultant fractions were tested for the inhibition of IL2 production in PMA stimulated EL4 cells (Fig. 3.14). All the inhibitory activity was recovered in fractions corresponding to high molecular weight. The factor could either be in an aggregated form, or associated with other macromolecules such as casein. Casein itself exists in calcium dependent micellar form in colostrum and milk (McMohan et al 1984) There was no difference in the elution pattern between acidified and nonacidified F2 human colostrum.

3.5. Transient acidification of colostrum increases its inhibitory activity

The two features of colostrum inhibitory factor namely the inhibition of IL2 production and its high molecular weight resemble features of the TGF β group of immunosuppressants. TGF β's are secreted in latent high molecular forms and get activated on transient acidification (Miyazono et al 1991). Therefore the the effect of transient acidification on the CIF activity was studied. Defatted, cell free, colostrum (F2) was transiently acidified to pH 3.0 for 5 minutes with 5N HCl and then neutralized with 5N NaOH and 1M HEPES, yielding thereby F2A. F2A had a greater inhibitory activity than F2 when assayed on PMA - stimulated EL4 cells (Fig. 3.15). Expressing inhibitory activity as ED_{50} units, where 1 ED_{50} U/ml induces 50% inhibition of IL2 production, F2 contained 120 U/ml, whereas F2A had an activity of 500 U/ml. F2A, like F2, was not toxic to any of the cell lines used in this work. Like F2, F2A actually increased the survival of T helper cell lines after exposure to IL2 inducing agents.

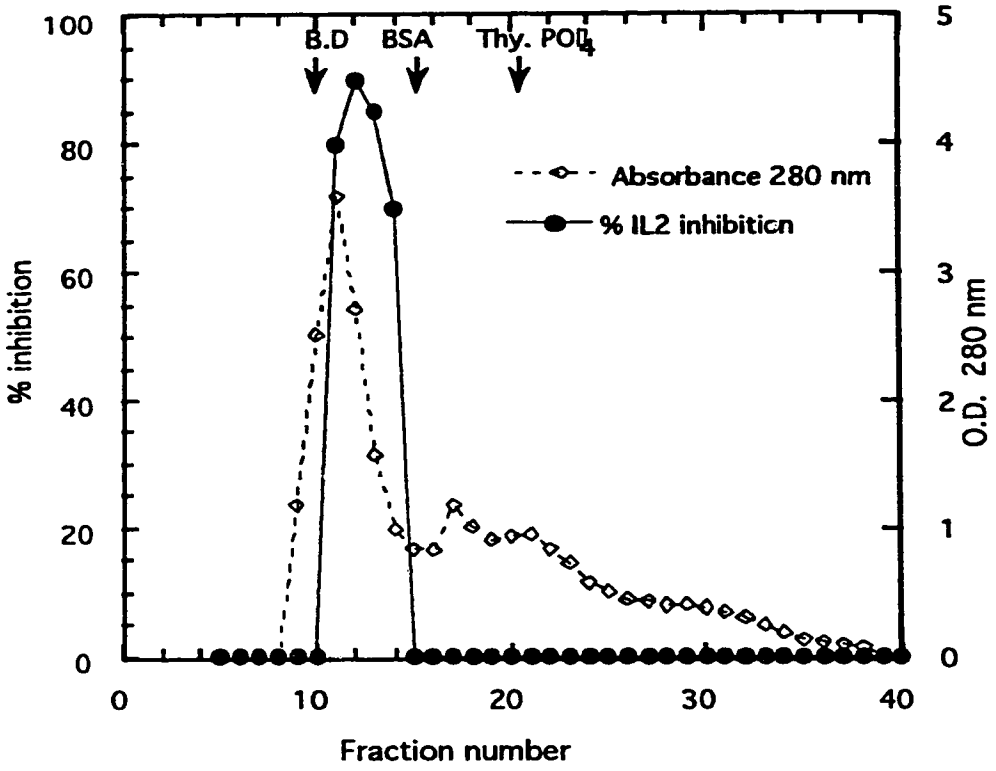

Figure 3.14: Gel filtration of human colostrum on a Sephacryl S-300 column. Total protein and percent inhibition of IL2 production by fractions of F2 colostrum were quantified. Molecular weight markers are blue dextran (B.D.; M_r, 2000 kD), BSA (M_r 68 kD), and thymidine phophate (M_r, 500 D).

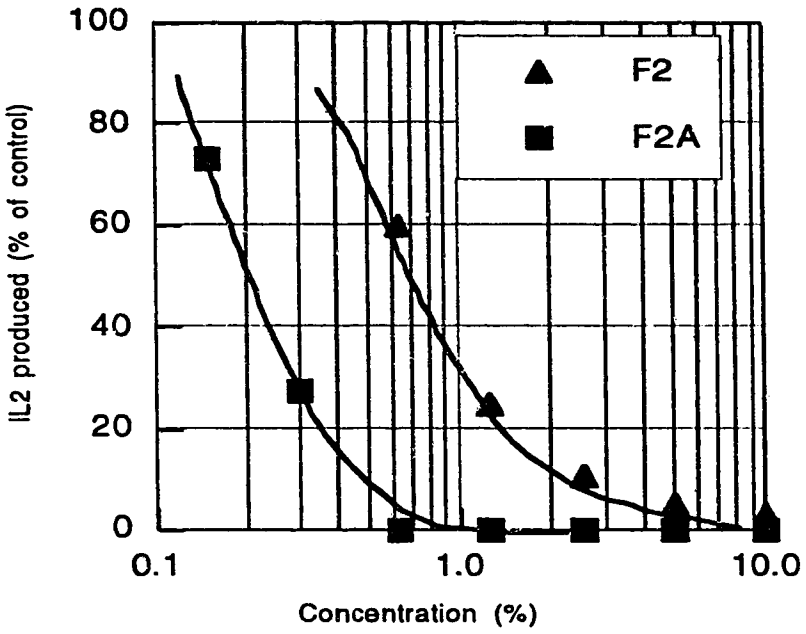

Figure 3.15: Effect of transient acidification of fraction 2 colostrum (F2) on its ability to inhibit IL2 production. F2 was acidified to pH3 for 5 minutes and neutralized to yield F2A. It was then added to the cultures of PMA-stimulated EL4 cells and IL2 production was measured as in Figure 3.4.

3.6. Effect of TGF β on the production of IL2 in PMA stimulated EL4 cells.

TGF β has been shown to inhibit IL2 production by an EL4 cell line (Espevik et al 1990). As shown in Figure 3.16 TGFβ1 added to PMA stimulated EL4 cells at the start of culture inhibited the production of IL2. There was 97% inhibition of IL2 production by TGFβ1 at a concentration of 5 ng/ml. This inhibition was apparent even at a concentration of 0.6 ng/ml TGFβ1. Stimulation of EL4 cells with ionomycin as well as PMA increased the IL2 response almost two-fold in the experiments of Figure 3.16. The response due to PMA alone, presumably acting as a protine kinase C activator was completely abolished by TGFβ1. However, the larger response due to addition of both stimulators was inhibited by just over 50%.

3.7 Effect of antisera to TGFβ on the colostrum inhibitory factor:

It would be reasonable to suppose that the ability of human colostrum to inhibit IL2 production is due to one or more TGFβ molecular species: TGFβ inhibits IL2 production by PMA stimulated EL4 cells, milk is known to contain TGFβ (Saito et al 1993), and the acid activation of CIF is reminicent of the ability of transient acidification to activate known TGFβ species. Therefore the ability of anti-TGFβ antibodies to inhibit IL2 production by human F2 colostrum was examined.

EL4 cells were stimulated with 10 ng/ml PMA in the presence of 10% F2A. Inhibition of IL2 production by F2A was examined in the presence of various concentrations of anti-TGFβ1 and -β2 antibodies added at the start of culture. Addition of anti TGFβ2 reduced the inhibitory effect of acidified colostrum by less than 10%, and anti TGFβ1 had even less effect (Fig. 3.17). A panspecific antiserum directed against a combination of TGFβ molecules (TGFβ 1, 1.2, 2, 3, and 5) had a similar effect on both F2 and F2A. In the absence of antibody F2 gave about 94% inhibition of IL2 production and F2A about 96%. As shown in Figure 3.17 and 3.18, addition of saturating levels of

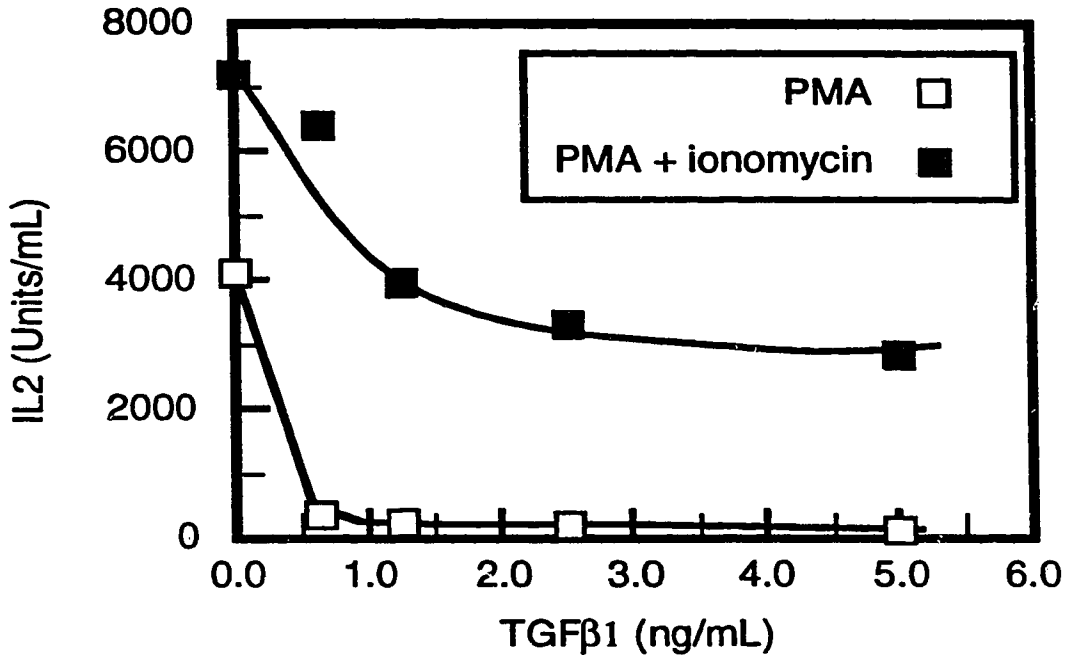

Figure 3.16: Effect of TGFβ1 on IL2 production in EL4 cells stimulated with PMA alone, or with PMA plus ionomycin . EL4 cells stimulated with 10 ng/ml PMA alone, or PMA plus 2 μM ionomycin, in the presence of indicated concentrations of TGFβ1. IL2 production was determined after 24 hours.

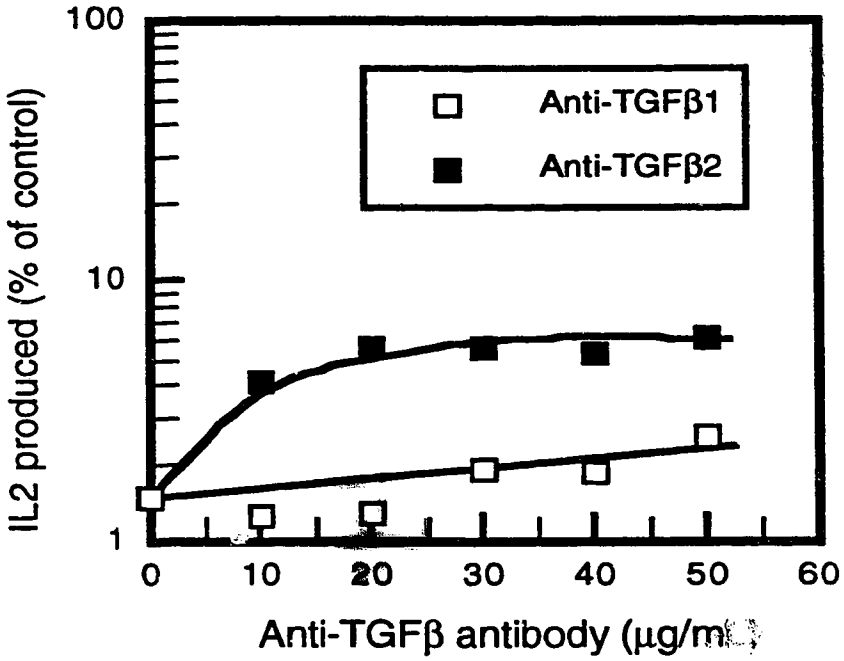

Figure 3.17: Effect of anti-TGFβ1 and -β2 antibodies on the inhibiton of IL2 production by human colostrum. F2A human colostrum was added at 1.25% to cultures of PMA-stimulated EL4 cells, together with indicated concentrations of anti-TGFβ antibodies. IL2 production was assayed after 24 hours, and is expressed relative to the control culture containing no F2A or antibodies.

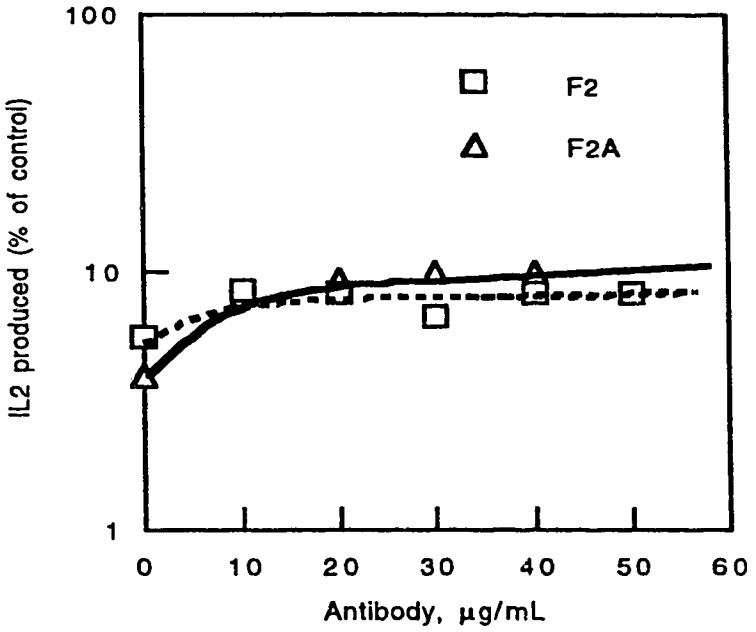

Figure 3.18: Effect of panspecific anti TGFβ antibody on the inhibition of IL2 production by human colostrum in PMA stimulated EL4 cells. F2 or F2A was present at 10% v/v, and various concentrations of panspecific anti-TGFβ antibodies were added at the start of the culture. IL2 production was assayed after 24 hours.

panspecific antibody reduced the inhibition by less than 10 % in both cases. As a control, panspecific antibody was found to inhibit the ability of recombinant human TGFβ2 (2 ng/ml) to inhibit IL2 production by PMA stimulated EL4 cells (Fig.3.19).

3.8. Colostrum inhibitory factor inhibits IL2 production more effectively than TGFβ

Although TGFβ blocked the production of IL2 by PMA stimulated EL4 cells almost completely, under conditions of stimulation with both PMA and the calcium ionophore ionomycin, inhibition by TGFβ molecules was only partial, between 30 and 50% whereas inhibition by F2 colostrum was essentially complete

Jurkat cells were stimulated with PMA (20 ng/ml) and ionomycin (2 μM). Various concentrations of TGF β1, -β2 or F2A were added at the start of culture. TGF β1 and -β2 inhibited IL2 production by less than 50%, whereas F2A inhibited the production of IL2 by Jurkat cells almost completely (Fig. 3.20 and 3.21). Similar results were obtained with EL4 cells as detected by RT-PCR (Fig.3.22). Addition of Ionomycin to the PMA stimulated cells led to about two-fold higher IL2 yield, but only half of this total was inhibitable by TGFβ1 or β2.

3.9. Bovine colostrum also contains colostrum inhibitory factor activity

Bovine colostrum was subjected to low speed centrifugation as described for human colostrum, to generate F2, and assayed for colostrum inhibitory factor. As shown in Figure 3.23a bovine F2 inhibited IL2 production by Jurkat cells stimulated with PMA + Ionomycin profoundly even at well below 1% by volume. Similarly, bovine F2 inhibited IL2 production in PMA stimulated EL4 cells (Fig. 3.23b). Calculated in ED_{50} units bovine F2 colostrum had ~ 400 u/ml of activity, or about four times more CIF activity than human colostrum. About 15% of the activity in bovine F2 colostrum was neutralized by panspecific anti-TGFβ antibodies (Fig. 3.28a).

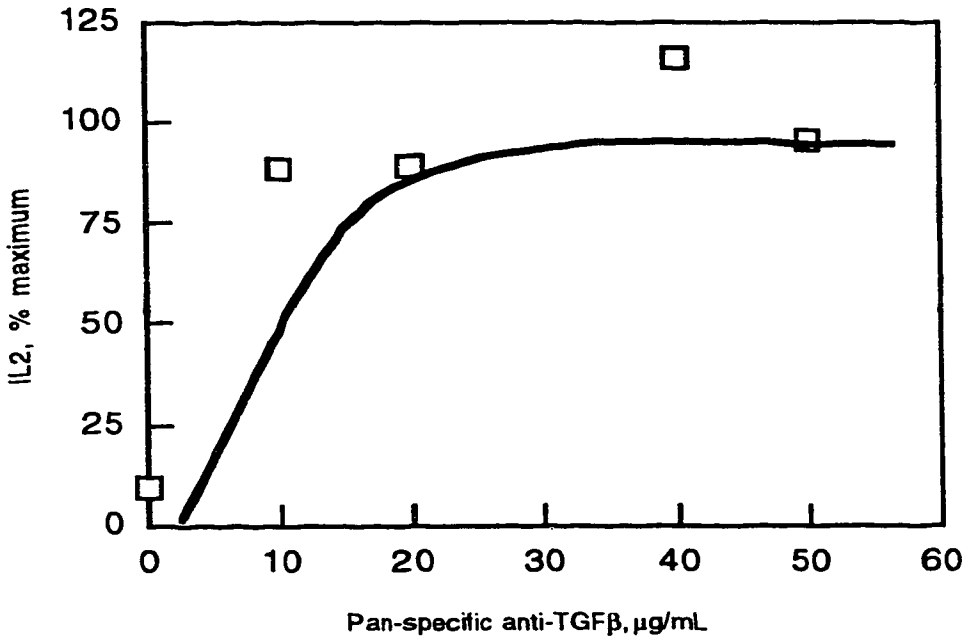

Figure 3.19: Effect of panspecific anti-TGFβ antibody on the inhibition of IL2 production by TGFβ2. PMA -stimulated EL4 cells were incubated with 2 ng/ml of recombinant human TGFβ2 and various concentrations of the panspecific anti-TGFβ antibody.IL2 was assayed after 24 hours. In this system (stimulation with PMA alone), TGFβ2 inhibited IL2 synthesis by more than 90%, and panspecific antibody blocked this inhibition completely.

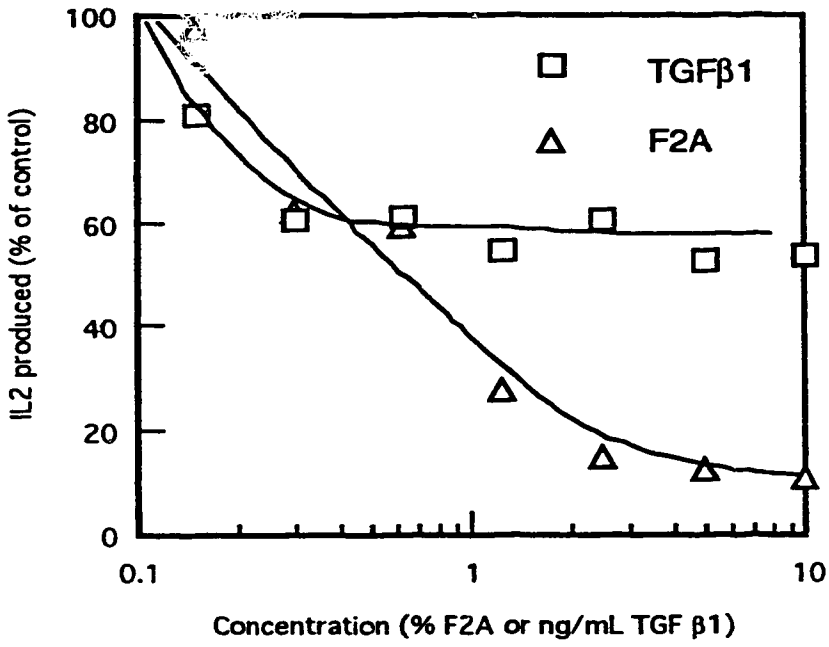

Figure 3.20: Effect of TGFβ1 and F2A human colostrum on IL2 production by Jurkat cells stimulated with PMA and ionomycin. Jurkat cells 2 x 10^6 /ml were incubated with 20 ng/ml PMA and 2 μM ionomycin and graded concentrations of recombinant human TGFβ1 or F2A. IL2 produced was assayed after 24 hours.

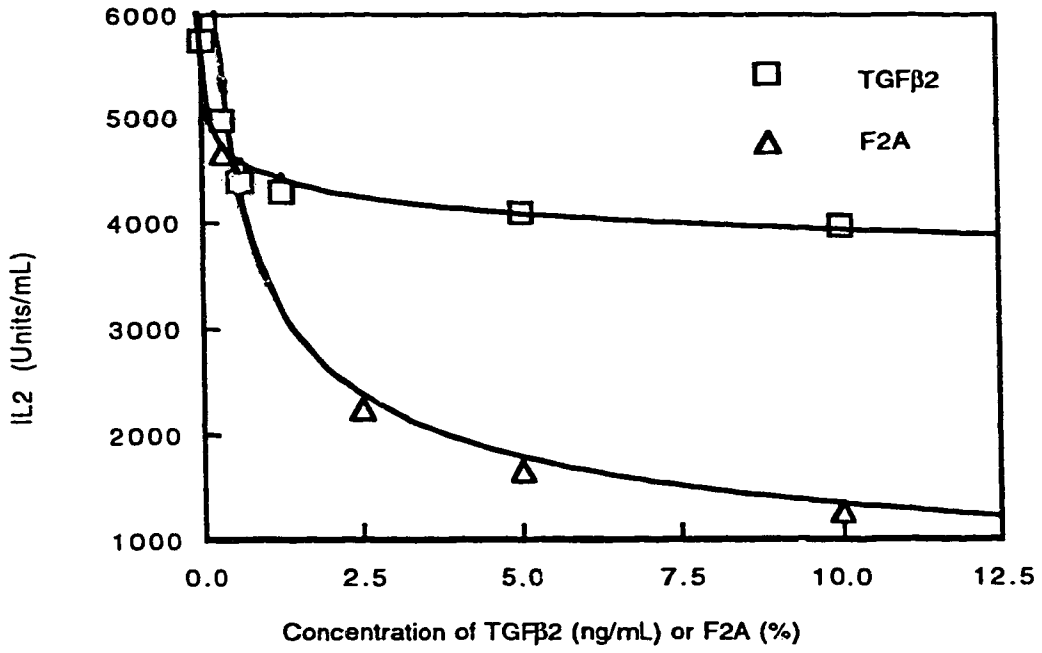

Figure 3.21: Effect of TGFβ2 and F2A human colostrum on IL2 production by Jurkat cells stimulated with PMA and ionomycin. Jurkat cells 2 x 10^6 /ml were incubated with 20 ng/ml PMA and 2 μM ionomycin and graded concentrations of recombinant human TGFβ2 or F2A. IL2 produced was assayed after 24 hours.

Figure 3.22: Effect of colostrum and TGFβ on the induction of IL2 mRNA in EL4 cells stimulated with PMA alone or PMA and ionomycin. IL2 mRNA was analysed after 18 hour culture of EL4 cells by the Reverse Transcriptase - Polymerase chain reaction (F3 is ammonium sulfate precipitated colostrum and F3A is acidified F3).

- Lanes 1- 6: EL4 cells stimulated with PMA (10 ng/ml). Additions to cultures:Lane 1- F2(10%), Lane 2-F2A(10%), Lane 3-F3(10%),, Lane 4-F3A(10%),, Lane 5-TGFβ1 (1 ng/ml), Lane 6- none, Lane 7-unstimulated cells.
- Lanes 8 - 13: EL4 cells stimulated with PMA (10 ng/ml) plus ionomycin (1.5 uM). Additions to cultures: Lane 8-F2(10%),, Lane 9- F2A(10%),, Lane 10-F3(10%),, Lane 11-F3A(10%),, Lane12-TGFβ1 (1 ng/ml), Lane 13-none, Lane 14-unstimulated cells.
- Lanes 15 to 18 experimental controls.

In PMA stimulated cells treated with colostrum (lanes 1 to 4) and TGFβ1 (lane 5) the accumulation of IL2 mRNA was inhibited.

In PMA and ionomycin stimulated cells treated with colostrum (lane 8 to 11) the accumulation of IL2 mRNA was inhibited but TGFβ treated cells (lane 12) did not inhibit the accumulation of IL2 mRNA.

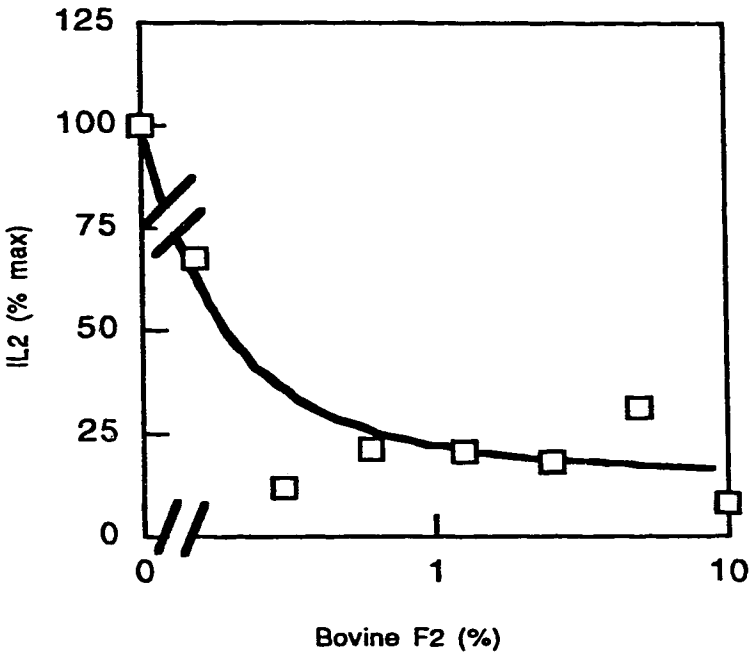

Figure 3.23a: Effect of F2 bovine colostrum on IL2 production in Jurkat cells stimulated with PMA and ionomycin.

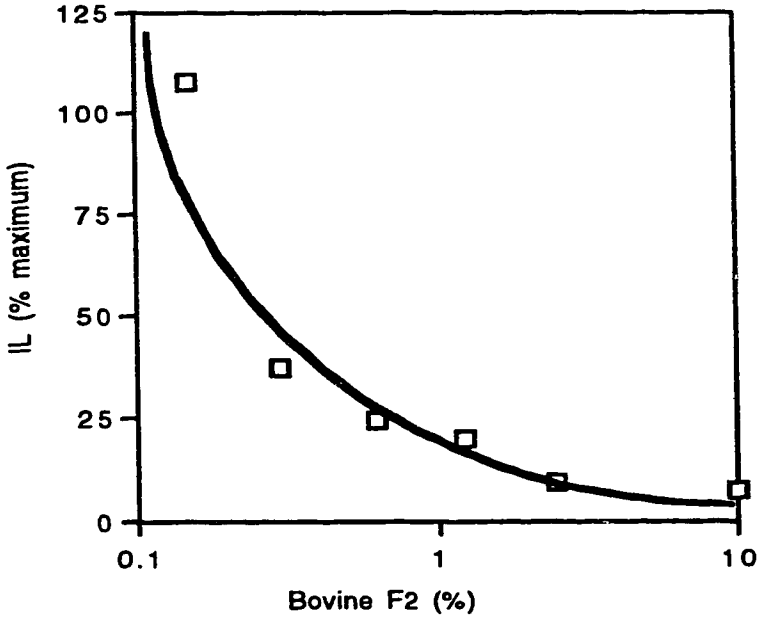

Figure 3.23 b: Effect of F2 bovine colostrum on IL2 production in EL4 cells stimulated with PMA.

3.10. Effect of bovine mature milk on the production of IL2 in Jurkat cells stimulated with PMA and ionomycin.

Homogenized bovine mature milk was treated in a similar manner as colostrum and tested for inhibitory activity in Jurkat cells stimulated with 20ng/ml PMA and 2μM ionomycin. Graded concentrations of F2 milk were added to the cells at the start of the culture. F2 milk even at a concentration of 10% had no effect on the production of IL2 in Jurkat cells (Fig. 3.24). Similarly, infant formula milk like Enfalac, Similac and Isomil had no inhibition of IL2 production as observed in T hybridoma cells stimulated with anti-CD3 (Fig. 3.25).

3.11. Separation of bovine colostrum into whey and casein fractions by acidification and calcium precipitation.

To study whether the inhibitory activity was associated with whey or casein fraction, colostrum was acidified to pH 4.3 and calcium was added to precipitate casein as described in chapter 2. As shown in Figure 3.26 most of the activity was recovered in the casein fraction.

Sucrose gradient centrifugation described by Patton et al (1985) was done to isolate fat globules from colostrum. The technique is described in Section 2.9. On sucrose gradient centrifugation of bovine F2 most of the activity was recovered in layer 4 which had the highest concentration of protein of the various layers. Transient acidification of bovine F2 increased its inhibitory activity from 476 U/ml to 909 U/ml (Fig. 3.27). Layer 4 was most activated by acidification and 35% of the activity in unacidified layer 4 could be neutralized by panspecific anti-TGFβ abtibodies (Fig. 3.28b).

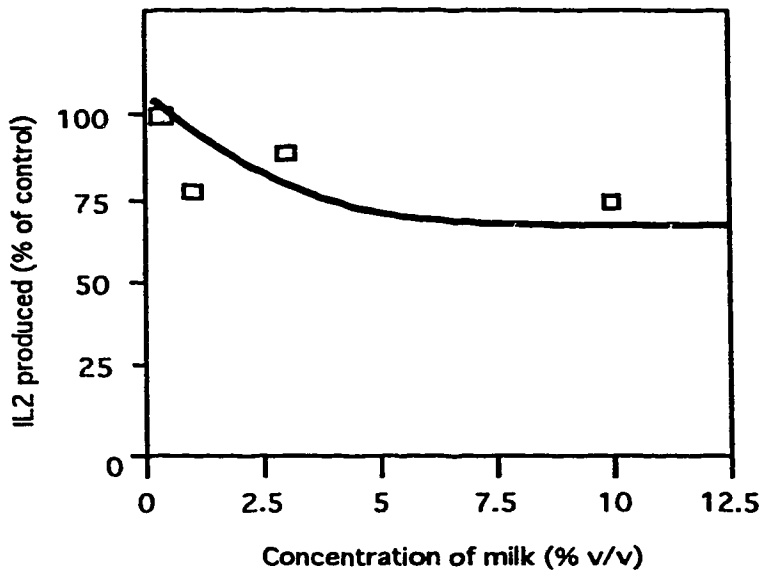

Figure 3.24: Effect of bovine mature milk on IL2 production in Jurkat cells. Jurkat cells were stimulated with PMA plus ionomycin as described in Figure 3.6. Various concentrations of F2 prepared from mature bovine milk was added at the start of the culture and IL2 was assayed after 24 hours.

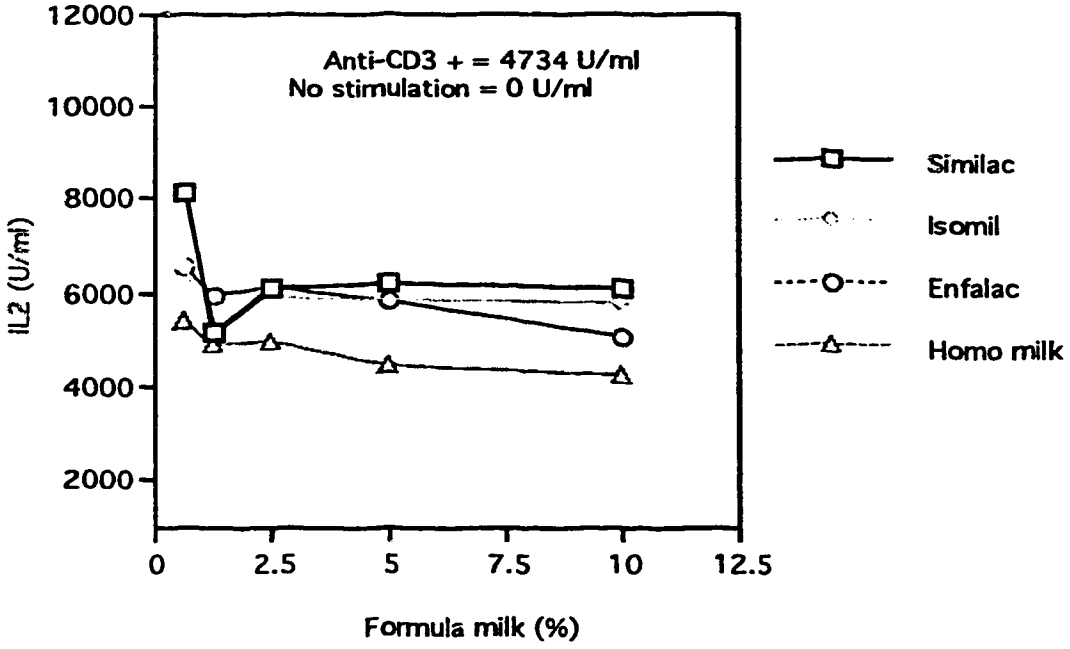

Figure 3.25: Effect of infant formula and bovine mature milk on IL2 production in anti-CD3 stimulated T hybridoma cells.

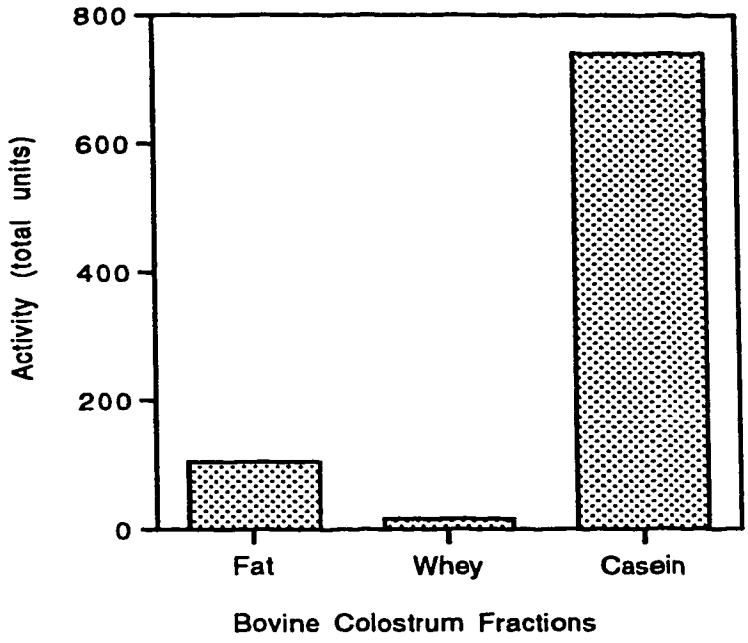

Figure 3.26: Effect of fat, whey, and casein fractions of bovine colostrum on IL2 production in PMA stimulated EL4 cells. F2 bovine colostrum was acidified to pH 4.3 with 6M HCl and left on ice for one hour. 2ml of 1.2M calcium chloride (final concentration of 20mM) was added to the above and left on ice for a further 10 minutes. The above treated colostrum was then centrifuged at 150,000 x g for 1 hour at 20^0 C, resulting in separation into a clear supernatant (whey), a precipitate (casein) and a fat layer on top. The casein fraction was reconstituted to the original volume by water. Each of these fractions were tested for the inhibition of IL2 production in PMA-stimulated EL4 cells. The inhibitory activity of these fractions was expressed in ED_{50} units, where 1 ED_{50} unit produces 50% inhibition of IL2 production.

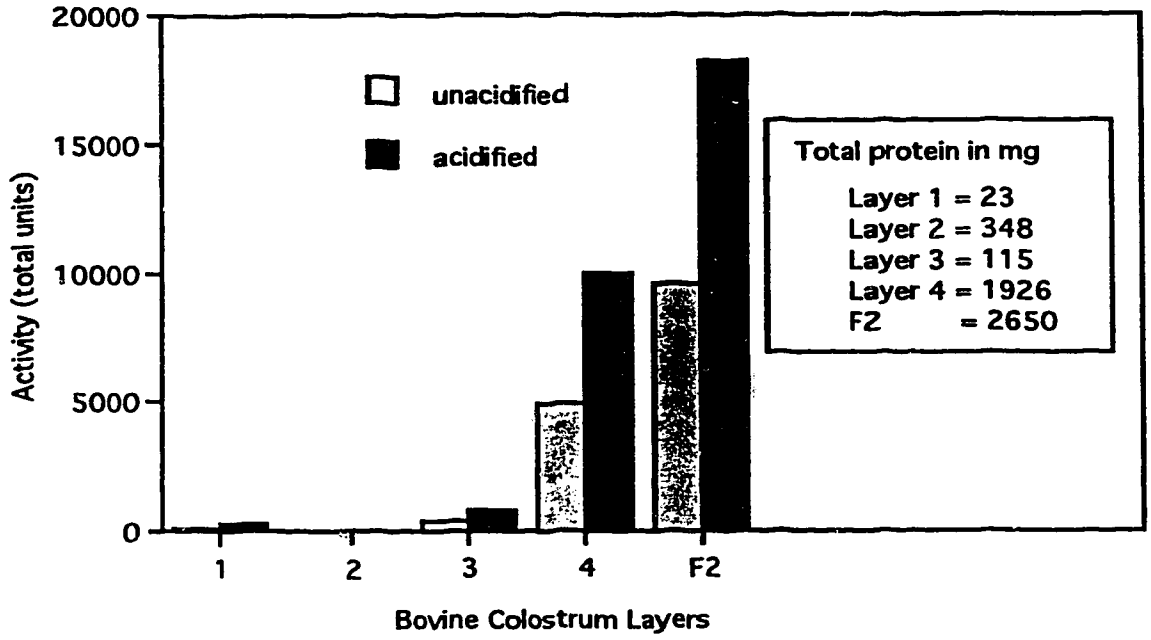

Figure 3.27: Effect of sucrose gradient centrifugation layers of bovine colostrum on IL2 production in PMA stimulated EL4 cells. On sucrose gradient centrifugation F2 bovine colostrum was separated into four layers. Layer 1 contained fat; layer 2 was PBS mixed with colostrum; layer 3 was at the interface; and layer 4 at the bottom contained the highest concentration of protein. Both acidified and unacidified layers were tested for the inhibition of IL2 production in PMA-stimulated EL4 cells. Most of the inhibitory activity (expressed in ED_{50} units) was recovered in the protein rich layer 4.

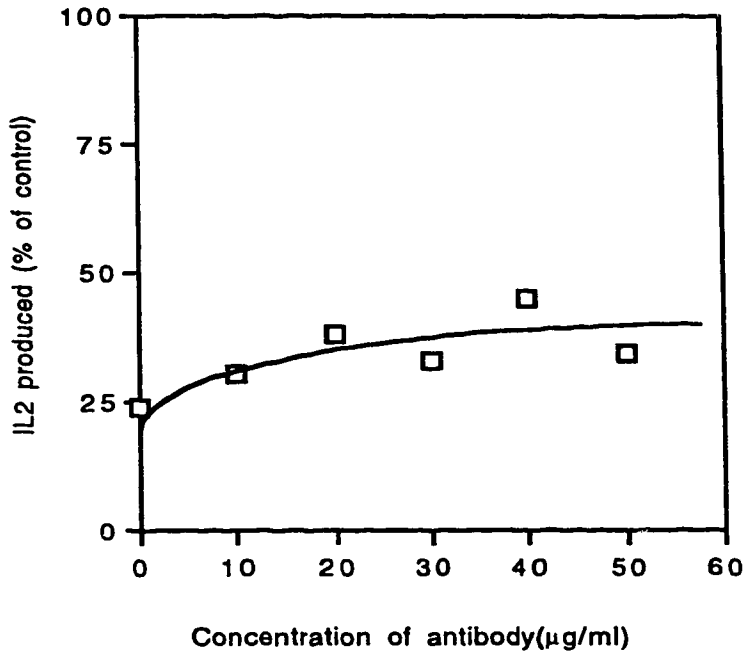

Figure 3.28a: Effect of panspecific anti-TGFβ antibody on the ability of inhibition of IL2 production by F2 bovine colostrum in 10 ng/ml PMA stimulated EL4 cells. F2 bovine colostrum was added at 5% v/v to the culture of PMA-stimulated EL4 cells, togeteher with the indicated concentrations of anti-TGFβ antibody. IL2 production was assayed after 24 hours, and is expressed relative to the control culture containing no F2 or antibodies.

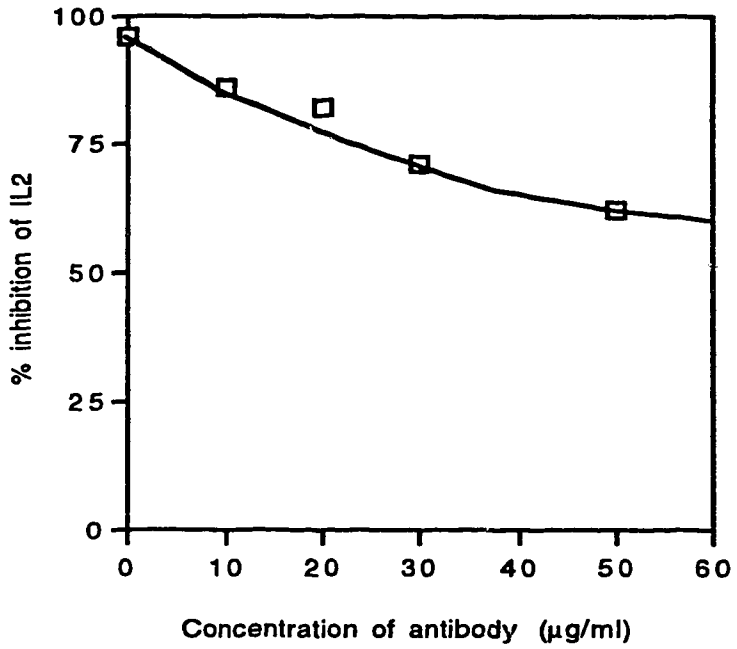

Figure 3.28b: Effect of panspecific anti-TGFβ antibodies on the inhibitory activity in casein fraction F2 bovine colostrum. Layer 4 of sucrose gradient layers was added at 5% v/v to the culture of 10 ng/ml PMA-stimulated EL4 cells, togeteher with the indicated concentrations of anti-TGFβ antibody. IL2 production was assayed after 24 hours, and is expressed relative to the control culture containing no F2 or antibodies.

3.12. Disruption of casein micelles by calcium chelation with EDTA.

Fifty mM $CaCl_2$ was added to F2 colostrum and centrifuged at 190,000 x g for 1 hour at 4^0 C to precipitate the casein as micelles. The resultant casein precipitate was dissolved in 200 mM EDTA (Griffin et al 1988) and dialyzed to avoid the toxic effects of EDTA on cells. This fraction was tested for inhibition of IL2 production in Jurkat cells stimulated with PMA and ionomycin. As shown in Fig. 3.29 the colostrum inhibitory factor coprecipitated with casein

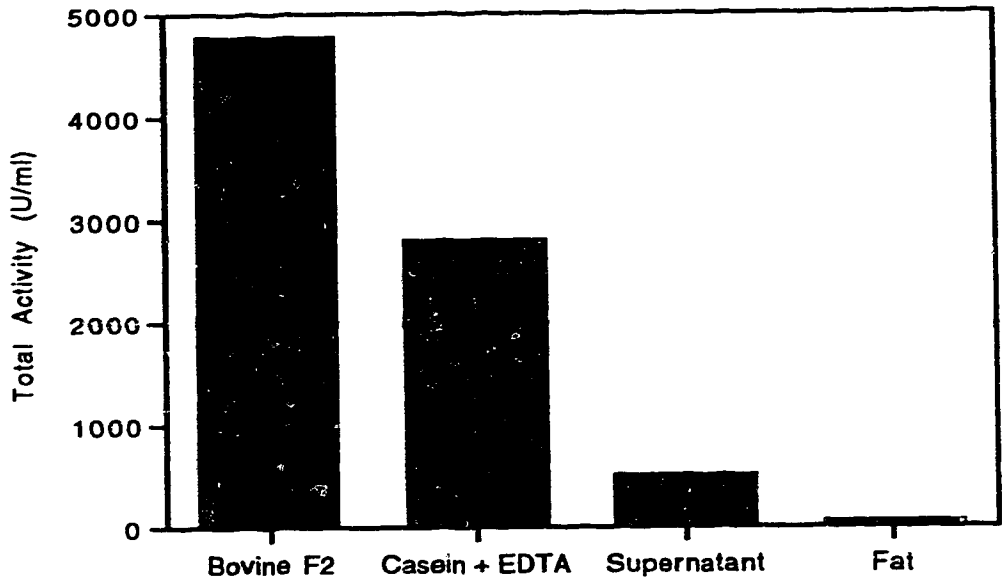

Figure 3.29: Effect of EDTA dissolved casein fraction on IL2 production in PMA stimulated EL4 cells.

4 DISCUSSION AND CONCLUSIONS

4.1 Discussion

Breast milk with its unique properties is of nutritional and physiological significance to the newborn. In particular, colostrum with its abundance of immunoregulatory proteins influences the postneonatal development of the mammalian immune system. Zimecki et al (1987) have suggested that the immunoregulatory proteins in breast milk act as buffers by adjusting the response to optimal levels, stimulating or inhibiting the immune response.

Immunoregulatory proteins such as secretory component and galactothermin influence the differentiation of B and T cell lineages (Zimecki et al 1987). Immunostimulating substances from delipidated human casein have been recognized by Jolles et al 1981. In addition, human milk and colostrum contain many lymphokines and growth factors, some of which are inhibitory in immune responses. This study concentrates on an immunosuppressant factor present in colostrum.

Colostrum contains more than one inhibitory factor that inhibits T cell function as measured by proliferation in lectin-stimulated or mixed lymphocyte assays. Colostrum was not toxic to any of the cell lines tested and did not affect autonomously growing cells. The inhibitory activity is therefore not a direct inhibitor of DNA synthesis or cell proliferation. Rather, it blocks the production of IL2 by stimulated cells in vitro. This study further characterizes this CIF and in particular demonstrates that it differs from known TGFβ molecules.

Colostrum inhibited the production of IL2 in a variety of T helper cell lines stimulated with lectins or antigens. Colostrum is an abundant source of glycoproteins, and glycoproteins by binding to lectins interfere with stimulation of cells. It might be of concern that CIF is simply the result of colostrum glycoproteins binding to, and blocking,

cell surface molecules that are necessary for stimulation by mitogens. To rule out this possibility 3 different types of T helper cell lines stimulated with different agents were used as IL2 producers (Figures 3.3, 3.4, 3.6, and 3.7). In the case of T cell hybridoma stimulation was through the antigen receptor, and inhibition probably cannot be accounted for by any effect on membrane surface hydrophobic molecules or by glycoprotein stimulation. Colostrum added to EL4 or Jurkat cells stimulated with PMA and calcium ionophores also showed the inhibitory effect. The various stimulating agents (PMA, ionomycin, and anti-CD3 antibody) are not thought to act in this way. Thus the inhibitory effect of colostrum is not due to the simple adsorption of stimulatory mitogens.

The inhibitory activity in colostrum is substantially free of low density lipids and cells and is prepared by low speed centrifugation of fresh colostrum (F2). Inhibition was apparent in all the human colostrum samples tested and the samples were thus pooled. The inhibitory activity is seen in colostrum and to a lesser extent in early milk. CIF has not shown species specificity in that the bovine factor works on human and mouse cells, and human material works on mouse and human cells more or less equally. Mature milk and commercially available cow's milk or infant formula milk did not inhibit the production of IL2 as tested in T cell hybridoma and Jurkat cells stimulated with PMA and ionomycin. Preliminary studies have shown that the inhibitory activity gradually disappears by day 35 postpartum. In contrast to colostrum not being toxic to cells, mature human milk between 2 and 5 weeks has been toxic to cells with or without stimulation. This toxicity is not due to complement mediated lysis as milk heated to 56^0 C for 30 minutes was still toxic to cells.

The increase in IL2 mRNA seen when EL4 cells are stimulated with PMA is blocked by CIF. In this aspect colostrum factor resembles cyclosporine A (CsA), although CsA was more potent in these studies (Figure 3.12).

On gel exclusion chromatography of F2 the activity eluted in fractions corresponding to high molecular weight. This suggests that the activity might either be in

an aggregated form or is associated with other macromolecules in colostrum. Transient acidification and reneutralization of colostrum increased its inhibitory activity 5 to 10 fold. F2 has 120 U/ml of inhibitory activity which increased to 500 U/ml on acidification. This suggests that acidification might release the active component of the factor. But gel filtration of acidified colostrum showed no difference in the elution pattern.

The fact that CIF is of high molecular weight and increases its activity on acidification suggests that it might belong to the TGF β group of immunosuppressants. TGF β is secreted in latent high molecular weight form, becoming activated on enzymatic treatment and extreme pH conditions. TGF β activity has been reported in human colostrum (Noda et al 1984) and milk (Saito et al 1993) and TGF β is known to inhibit IL2 production by IL1 β stimulated EL4 NoB-1 murine thymoma cells (Espevik et al 1990). Bovine milk growth factor (MGF), has been shown to inhibit Con A induced proliferation of PBMC and also IL2 and IL4 dependent proliferation of human T cells (Stoeck et al 1989). It was later shown by Jin et al 1991 that MGF was a mixture of TGF β1 and β2. From the results presented here CIF is not due to TGF β. There are two kinds of basic differences between known members of the TGF β family and CIF of colostrum. Firstly, antibodies that neutralize known TGF βs did not block a significant portion of the inhibitory activity in colostrum. Although some TGF β activity, particularly β2 was present in human colostrum, it accounted for only 10% of the inhibitory activity (Figure 3.17). The rest of the activity in human colostrum was unaffected by pan specific anti TGF β antibodies directed against TGF β1,β2, β1.2, β3, and β5 (Figure 3.18). It could be argued that TGF β may exist in a latent form in colostrum and thus is not available to the antibody. But the bulk of IL2 inhibitory activity in acidified colostrum was also not affected by panspecific anti TGF β antibodies. Secondly, the inhibitory factor of colostrum differs from known TGF βs in the mechanism of action. This is evident in experiments in which either EL4 or Jurkat cell lines were stimulated with PMA and calcium ionophore. F2 colostrum inhibited IL2 production essentially completely,

whereas TGF β1 and -β2 inhibited less than 50% even at saturating levels (Figures 3.20, 3.21). Addition of ionomycin increased IL2 production two fold, but less than half of this was inhibitable by TGF β. These findings were also observed at the level of IL2 mRNA (Figure 3.22). TGF β inhibits the induction of IL2 mRNA in PMA stimulated EL4 cells but IL2 mRNA was inhibitable only to the extent of 30% to 50% when PMA and ionomycin were used to induce the accumulation of IL2 mRNA. The upstream region of the IL2 gene contains several sites that are targets for inducible transcriptional activators. Some of these activations are sensitive to the immunosuppressant CsA, which effectively blocks IL2 transcription. The major inhibitory site of action of TGF β in the activation of the IL2 gene of PMA-stimulated EL4 cells has been mapped to the Upstream Promoter Site, centered around position -80 relative to the transcriptional start site (Brabletz et al 1993), whereas CsA acts primarily, although not exclusively, by blocking the activation through the NF-AT site lying at -275 (Emmel et al 1989). This is probably the basis for the more complete inhibition of IL2 transcription by CsA, compared to TGF β. The extent of inhibition by CIF is more similar to that of CsA than it is to TGF β. Thus colostrum factor, differs from the known TGF βs in its structure and mode of action.

Cortisol is present in human colostrum and milk and can cause similar inhibition of IL2 synthesis (Gillis et al 1979). Several lines of evidence suggest that the inhibitory activity in colostrum is not cortisol. Firstly, the two cloned T lymphocyte cell lines, EL4 and Jurkat, used to assay inhibitory activity are insensitive to dexamethasone (Arya et al 1984, Culpepper et al 1985). For Jurkat cells this has been shown to be due to lack of glucocorticoid receptors (Vacca et al 1990,1992). Secondly, the concentration of cortisol in colostrum is unlikely to inhibit the production of IL2 by T cells. The median cortisol level measured during the first 24 hours postpartum in human colostrum is about 60 nM (Saulski et al 1981). Human colostrum inhibits 50% of IL2 produced by Jurkat cells at 0.7% by volume, which corresponds to a cortisol concentration of about 0.4 nM. But in the case of dexamethasone it requires a concentration of 3 nM to inhibit 50% of IL2

produced in primary T cells. Finally, dexamethasone suppresses IL2 produced in primary T cells stimulated with PMA plus either anti-CD3 or anti-CD28 antibodies, but not if the stimulation is with PMA plus ionomycin (Furue et al 1991). Thus the above factors argue against the fact that CIF in colostrum is cortisol.

To further characterize the CIF bovine colostrum was studied. Bovine colostrum was treated like human colostrum by low speed centrifugation and assayed for inhibitory activity. Bovine F2 inhibited the production of IL2 by Jurkat cells stimulated with PMA and ionomycin as well as by EL4 cells stimulated with PMA. It was four times more active than human colostrum having 400 U/ml of activity. Transient acidification and reneutralization increased its activity and a significant portion (more than 60%) of this activity could not be blocked by panspecific anti TGF β antibodies.

Fractionation of bovine F2 into casein, whey and fat was done by acidification and calcium precipitation followed by centrifugation at 190,000 x g for 1 hour. This procedure precipitates up to 90% of the casein (Kunz et al 1989). Casein, whey and fat fractions were assayed for inhibitory activity. Most of the activity was associated with the casein fraction (Fig.3.26). Sucrose gradient centrifugation of bovine F2 yielded 4 layers. Most of the activity was recovered in the protein rich layer 4. It was layer 4 which was activated on acidification (Fig. 3.27). The bulk of activity in this layer could not be neutralized by anti TGF β antibodies. To separate CIF from colostral casein, the casein precipitate obtained after centrifugation was solubilized by adding EDTA. EDTA chelates calcium and thus disrupts casein micelles. CIF coprecipitated with casein. It seems unlikely that the activity resides in the bulk of casein itself as mature milk contains a high concentration of casein but not the CIF.

The above findings suggest the presence of a novel immunosuppressant in colostrum that coprecipitates with casein. Its function is not clear. It might play an important role in preventing allogeniec reactions of the graft versus host type (Hooton et al 1991). There is substantial evidence that breast milk contains immunoactive cells

including macrophages, B cells and T cells (Crago et all 1979) and human milk and colostral lymphocytes have been demonstrated to proliferate in response to alloantigens (Parmely et al 1977). The immunosuppressant factor might prevent the immunoactive T cells present in colostrum from reacting against the paternal antigens present in the neonate. IL2 is an important cytokine in T cell dependent immune responses and its inhibition by colostrum suggests that colostrum may have a profound effect on the development of the immune response to T-dependent antigens. It therefore may have a strong influence on the immune system of the infant. Mincheva-Nillson et al (1990) have postulated that the inhibitory activity down- regulates the immune response to environmental antigens in the neonate until T suppressor activity develops thereby preventing atopic reactions to these antigens later in life. It could also be that the T-cell inhibitory activity acts on the lymphocytes in the intestinal wall of the infant (gut associated lymphoid tissue, GALT) and prevents hyperactive responses there, amongst others against food antigens, reducing hypersensitivities and food allergies. The inhibitory factor causes the suppression of T lymphocyte mediated immune responses within the first few weeks after birth. It is during this period that important cell membrane structures, such as T cell receptors become fully expressed (Pilarski et al 1991). An immunosuppressant factor in colostrum would help to regulate the immune system of the neonate at this critical period of self-nonself discrimination allowing for activation against environmental antigens, but unresponsiveness to autoantigens. The clinical findings of improved immune responses to vaccination and decreased autoimmunity like IDDM and chronic inflammatory condition like Crohn's in breast fed children may be attributable to this immunosuppressant effect of colostrum, in effect providing immuno-regulation.

Colostrum factor blocks the expression of the IL2 gene by affecting both the protein kinase 'c' and the calcium pathway. In this mode of action colostrum factor

resembles CsA. However, in contrast to CsA, colostrum factor does not have the toxic effects of CsA and could be used as an immunosuppressant drug with less side effects.

4.2 Conclusions

1. Defatted, cell free human colostrum (F2) contains more than one factor inhibiting the production of interleukin 2 in various types of T helper cell lines stimulated with different agents. F2 at 10% by volume is not cytotoxic to any of the cell lines tested and does not affect the growth of autonomously growing cells. The factor therefore, is not a general inhibitor of DNA synthesis. The inhibition is specific to colostrum and early milk and is not seen in mature milk.
2. F2 human colostrum inhibits the accumulation of IL2 mRNA normally seen when EL4 cells are stimulated with PMA. In this aspect F2 acts similarly to cyclosporine A, though it is not as potent in these studies.
3. The inhibitory factor has a very high molecular weight, suggesting aggregation or association with other macromolecules in colostrum.
4. Transient acidification and reneutralization of F2 increases its ability to inhibit IL2 production 5 to 10 fold . F2 has 120 U/ml of activity which increased to 500 u/ml on acidification.
5. TGF β2 accounts for about 10% of the inhibitory activity in human colostrum. The rest of the activity does not belong to any of the known TGF β's as panspecific anti TGF β antibodies does not block a significant portion of the inhibitory activity.
6. The inhibitory factor of colostrum behaves differently from the known TGF β's in its mode of action. Colostrum factor inhibits IL2 production more effectively than TGFβ in Jurkat and EL4 cells stimulated with PMA and calcium ionophore. This suggests that colostrum factor inhibits both the calcium signalling and signalling through the protein kinase 'c' pathway whereas TGF β does not. Thus colostrum factor differs from the TGFβ group of immunosuppressants in its structure and mode of action.

7. Bovine F2 also inhibits the production of IL2 in Jurkat cells stimulated with PMA and ionomycin and in EL4 cells stimulated with PMA. Bovine F2 has 400 U/ml of inhibitory activity which is four fold more active than human colostrum.
8. Bovine F2 can be activated by acidification and the resulting IL2 inhibitory potential is only partially sensitive to anti-TGFβ antibodies as in the case of human F2.
9. Most of the activity in bovine F2 can be recovered in the casein fraction. The factor coprecipitates with casein after disruption of casein micelles with the addition of EDTA.

The above results therefore suggest the presence of a novel immunosuppressant factor in human and bovine colostrum that inhibits the production of interleukin 2 and does not belong to any of the known TGF β group of immunosuppressants. The factor coprecipitates with casein.

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