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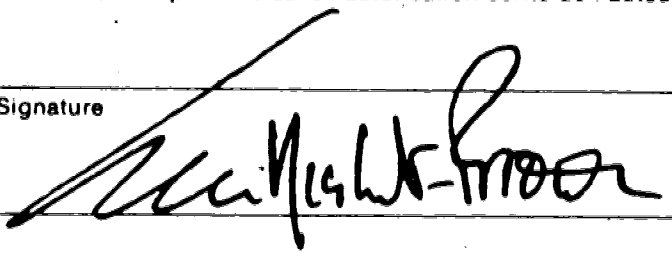

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by

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Abstract

This thesis describes studies undertaken to investigate the role of the major histocompatibility complex in governing cell-cell interactions in the immune response.

A macrophage-dependent *in vitro* T cell proliferation assay was used as a first approach to examine the interaction between primed T cells and APC. With this system, we showed that immune response (*Ir*) genes are expressed by the antigen presenting cell in responder and nonresponder strains to the synthetic peptide antigen, TNP-18, with heterozygous (R x NR) responding T cells, and R or NR APC. Alloreactivity between nontolerant homozygous T cells and fully histoincompatible APC prevented us from looking directly for H-2 restriction of the T cell - APC interaction.

To overcome this problem, we turned to tolerant T cells from parent -> F_1 foetal liver chimaeras, finding persistence of H-2 restriction between APC and T cells, in the proliferation assay, when alloreactivity is eliminated. This, together with others' results, excludes that the apparent H-2 restriction seen under some circumstances for nontolerant T cells necessarily reflects negative or suppressive allogeneic effects between the cell populations.

We also investigated the ability of nonspecific macrophage factors to replace APC in the T cell proliferative response, showing that they are apparently able to overcome H-2 restricted and *Ir* gene controlled antigen presentation *in vitro*. This led us to postulate the existence of two pathways of T cell activation by antigen, one H-2 restricted or *Ir* controlled with an absolute APC dependence, which operates in the presence of limiting amounts of antigen; and the other, perhaps nonphysiological, requiring high concentrations of soluble antigen and nonspecific APC derived factors. The lack of H-2 control seen under these conditions may reflect direct T cell triggering by high antigen concentrations.

In an extension of these experiments, we looked at the H-2 restriction patterns of T cells derived from two-way foetal liver chimaeras *A* + *B* -> (*A* x *B*)F_1, or tetraparental mice *A* <-> *B*. Against our initial expectations, homozygous T cells from the *in situ* primed FLC showed strict self-preference in the proliferation assay, cooperating only with syngeneic APC. Studies with the limited number of tetraparental animals available so far indicate, however, that T cell cooperative preferences can be modulated

away from 'self'. Two patterns of cooperativity appear to exist in these animals; unrestricted cooperation seen in 'balanced' chimaeras; and restriction to the predominant somatic haplotype, seen in 'unbalanced' animals.

The means by which restriction is imposed in the animal, and the reasons for the clear difference between stem cell chimaeras and tetraparentals are still unresolved. We feel, however, that the restriction pattern seen for cells from two-way FLC may be independent of the chimaeric host haplotype, and of priming, perhaps reflecting 'learning' experiences of the stem cells in the embryonic donor environment. By contrast, such learning would not yet have occurred in the eight-cell embryos used for constructing tetraparentals; hence, cells from these animals would show cooperative preferences dictated solely by the milieu in which differentiation occurs.

Finally, we examined MHC restriction of the interaction between T cells, B cells, and APC *in vivo*. To obviate the potential for alloreactivity between any or all of the cooperating cell populations, we used T and B cells taken from parent -> F_1 foetal liver chimaeras. Under these conditions, we showed a requirement for B cell - host syngenicity in the *in vivo* response to the thymus dependent antigen, TNP-KLH. These studies do not, however, exclude the possibility that the adoptive host may contain residual radioresistant alloreactive T cells able to suppress the function of the transferred T or B cells by a nonspecific allogeneic effect.

To address this point, we repeated our earlier experiments, comparing tolerant (chimaeric) hosts and normal animals; both were equivalent. Thus, we feel suppression is inadequate to account for our observations. Host - B cell restriction has not been previously shown for TD antigens, or *in vivo*; prior studies have relied on TI antigens, in *in vitro* culture systems. Rather puzzling is our failure to find the T cell - host restriction which has been almost universally reported by other groups. Our results exclude that the apparent lack of restriction is due to the failure to prime adequately, and strongly suggest that it does not reflect priming in the context of nonrestricting (F_1) APC. It should be emphasized, however, that irrespective of the reasons for this apparent lack of restriction, it is only under these conditions of unrestricted helper T cell function that APC - B cell restriction can be shown.

Preface

This dissertation describes a series of studies on the control by the major histocompatibility complex (MHC) of cellular interactions in the generation of immune responses *in vitro* and *in vivo*.

The results in Chapter IV have been published in *Immunogenetics*. These studies were carried out in collaboration with Dr. K.C. Lee, and Dr. T.G. Wegmann.

The work described in Chapter VI has been accepted for publication in *The Journal of Experimental Medicine*. The studies appearing as Appendix I are in press in the *Proceedings of the National Academy of Sciences*, and were done in collaboration with Dr. T.G. Wegmann.

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List of Abbreviations

Immunoglobulins and chemical compounds are named in accordance with the WHO and IUPAC nomenclatures, respectively. Other abbreviations and symbols used include:

ABC	antigen binding cell
AFC	antibody forming cell
APC	antigen presenting cell
ATS	anti-thymocyte serum
aTx	adult thymectomized
B-cells	non-thymus derived cells
BA	*Brucella abortus*
BM	bone marrow
BRBC	burro red blood cells
BSA	bovine serum albumin
C'	complement
CFA	complete Freund's adjuvant
CFU-S.	colony forming unit - spleen
CI	cell interaction (molecules)
CMJ	corticomedullary junction
Con A	Concanavalin A
CRIA	cellular radioimmunoassay
CTL	cytotoxic T lymphocyte(s)
DTH	delayed type hypersensitivity
FCS	foetal calf serum
FL	fluorescein
FLC	foetal liver cells/chimaeras
GALT	gut-associated lymphoepithelial tissues
GAT	$Glu^{60}Ala^{30}Tyr^{10}$
GLϕ	Glu-Lys-Phenylalanine
GRF	genetically related factor
GvH	graft *versus* host

H-2	histocompatibility-2 locus
(H,G)-A--L	(His,Glu)-Ala--Lys
Ia	I-region associated
Ig	immunoglobulin
Ir	Immune response gene
KLH	keyhole limpet haemocyanin
L-15	Leibowitz L-15 medium
LDH	lactate dehydrogenase
LPS	bacterial lipopolysaccharide
Lyb	B lymphocyte alloantigen
Lyt	T lymphocyte alloantigen
MBSA	methylated bovine serum albumin
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MØ	macrophage
NA	nonadherent
Nase	Staphylococcal endonuclease
NMF	nonspecific macrophage factor
NR	nonresponder
nTx	neonatally thymectomized
NWF	nylon wool filtered
OVA	ovalbumin
PBS	phosphate buffered saline
PEC	peritoneal exudate cells
PFC	plaque forming cells
PHA	phytohaemagglutinin
PLL	poly-L-lysine
Poly-18	$(Glu-Tyr-Lys-(Glu-Tyr-Ala)_3)_n$
PPD	purified protein derivative of tuberculin
PTP	postthymic T cell precursor

PWM	pokeweed mitogen
R	responder
RE	reticuloendothelial
RFC	rosette forming cell(s)
RGG	rabbit gamma globulin
RPMI-1640	RPMI-1640 tissue culture medium
SE	standard error of the mean
S/N	supernatant
SRBC	sheep red blood cells
T-cells	thymus derived lymphoid cells
TD	thymus-dependent (antigen)
TDL	thoracic duct lymphocytes
(T,G)-A--L	(Tyr,Glu)-Ala--Lys
theta	Thy 1 alloantigen
Thy 1	Thy 1 alloantigen
TI	thymus-independent (antigen)
TL	thymus leukaemia (antigen)
TNP	trinitrophenyl derivative
TNP-18	[Glu-Tyr-Lys(TNP)(Glu-Tyr-Ala)$_3$]
Tx	thymectomized
WK-14	Whitten's WK-14 embryo culture medium

I. Introduction

The cellular interactions involved in the generation of an immune response, and the controls acting on these interactions, have been a major preoccupation of cellular immunology for over a decade.

The objective of this chapter is to review the literature pertaining to this area, and more particularly to my research, and to attempt to place the development of this field in an historical context. Much of the work which has been done in this area is related, directly or otherwise, to the ontogeny of the immune system, and these aspects will consequently form the major focus of this section.

A. Functional organization of the immune system

One of the fundamental advances in cellular immunology during the last two decades was the realization that the differentiation of antigen-reactive lymphocytes can follow either of two independent pathways, one dependent on the thymus, and the other, not.

In outline, the ontogenic sequence involves migration of stem cells from the haemotopoietic tissues (foetal liver and yolk sac in the embryo; bone marrow in the adult) to the primary lymphoid organs, the thymus, and the bursa of Fabricius (bursa-equivalent in mammals). These organs are sites of intense antigen-independent cell proliferation and differentiation, and the particular microenvironments of these organs strongly influence the functional capabilities of the cells emerging from them. The mature (or relatively so) cells emerging from the primary organs pass to the peripheral or secondary lymphoid organs - spleen, lymph nodes, Peyer's patches, and blood - where the antigen-dependent immunological functions of the cells are expressed. Genetic influences imposed on the cells in the primary lymphoid organs are most frequently manifested at the level of the secondary organs, when functional immunocompetence is achieved.

B. Ontogeny of T Lymphocytes

In this section, we propose to examine in some detail the ontogeny of T and B cells, with particular emphasis on the kinetics of acquisition of immunological functions in intact foetal and neonatal animals, and in stem cell reconstituted adult animals.

Origins of stem cells

Development of a functional T lymphocyte system is dependent on the presence of the thymus. Early studies by Verdun (1898) established that the thymic rudiment is derived from the III and IV pharyngeal pouches, and is of ectodermal origin, although it requires the presence of endoderm for development (Hammond, 1954). The further development of the lymphoid thymus has been a point of controversy for over a century. The transformation hypothesis of Kolliker (1879) and others proposed that the thymocytes, Hassall's corpuscles, and thymic reticulum arise by differentiation from epithelial elements. Early studies tended to support this view (Beard, 1900; Bell, 1906), and recently Ackerman (1967) and others have claimed to find transitional forms between thymic epithelial and lymphoid cells. Curiously, Ackerman has also published work compatible with a substitution hypothesis (*vide infra*), but fails to attempt to reconcile the discrepancy (Ackerman and Hostetler, 1970).

By contrast, the substitution hypothesis of Hammar maintains that thymic lymphocytes derive from connective tissue lymphocytes which migrate into the thymic anlage. Maximow's studies (1908, 1912) were consistent with the Hammar theory, and further suggested that the lymphocyte precursors arise from mesenchymal cells which undergo differentiation into large basophilic amoeboid haemocytoblasts, before migrating into the thymic epithelium. Other workers have obtained evidence consistent with this view (Norris, 1938), and this seems to be the most satisfactory model at present.

Various modifications of these theories have been proposed, and among these, perhaps the strongest support for a transformation-like hypothesis came from the work of Auerbach (1961). Pre-lymphoid (12 - 12-1/2 day) mouse thymic anlage was transplanted to the chorioallantoic membrane of the chick embryo, and subsequently showed only mouse lymphoid development. Auerbach interpreted these results as indicating epithelial to lymphoid transformation. Moore and Owen (1967) obtained similar results, but also showed conclusively the presence of large basophilic cells with

prominent nucleoli in eleven day mouse embryo thymic rudiments, large numbers of these cells being found in twelve-day thymus. These cells closely resemble the putative lymphocyte precursors of Maximow. Together, then, these studies show that lymphocyte development occurs from stem cells already present in the anlage by twelve days of gestation, without addressing their origin.

Several approaches have been taken to determine the origin of these basophilic cells, usually in the chick embryo. Moore and Owen (1967a) used a sex chromosome marker system in pairs of embryos with vascular anastomosis produced by parabiosis. Embryos with early yolk-sac anastomosis (prior to the development of thymic lymphopoiesis) showed high levels of thymic chimerism, while embryos in which anastomosis of the chorioallantoic vessels was established later in ontogeny showed only minimal thymic chimaerism, while marrow, spleen, and bursa were markedly chimaeric. The haematogenous theory of thymic lymphocyte origin, basically a modification of the substitution theory, was formulated to account for these results. Briefly, it recognizes that the thymic anlage is populated by cells present in the surrounding mesenchyme, but extends this in considering these cells to have migrated there via the blood stream, rather than arising by mesenchymal transformation *in situ*.

This hypothesis was tested in two ways, one involving the grafting of chick or mouse thymic rudiments onto the CAM, and using sex chromosome or metaphase patterns to determine the relative host and donor contributions to the thymic population (Moore and Owen, 1967b). Analogous to the earlier results, the early chick thymus was almost completely repopulated with host-derived cells; while later grafts showed mixed host-donor chimerism. The repopulation of mouse thymus showed species specificity, with early rudiments failing to show lymphoid development, and later (12 - 12-1/2 day) grafts developing normal thymic architecture, with distinct cortical and medullary patterns. Studies by Owen and Ritter (1969) reinforced this conclusion.

The role of the basophilic cells as lymphoid precursors was directly shown by Moore (1971), again in the chick. Tritium-labelled embryonic haemopoietic cells were injected into developing chick embryos, and the fate of the labelled cells followed autoradiographically. The morphology and initial migratory kinetics of the cells was characteristic of that of the large basophils; subsequently, label was detected in cells

morphologically similar to medium and small thymic lymphocytes.

Intrathymic and extrathymic maturational events

The thymus is continuously repopulated by stem cells during adult life, although comparatively little is known of this process. Irradiated mice can be protected by infusion of yolk sac, foetal liver, or adult bone marrow cells, suggesting that the stem cell sources are generally equivalent (Stutman and Good, 1971), although Stutman (1977) has presented evidence that yolk sac cells require maturation in the bone marrow environment to become competent to repopulate the thymus. The process of intrathymic and extrathymic maturation of T cells remains of great interest to-day. Morphological studies have been generally unrevealing, and most work has in consequence centred on autoradiographic and chromosome marker studies, and on the acquisition of cell surface markers.

Histologically, the thymus comprises a highly cellular and actively mitotic cortex, and a relatively amitotic medulla. The blood supply to the organ is remarkable. Arterioles enter the thymic substance at the corticomedullary junction, and give off capillary arcades into the cortex; these anastomose extensively under the capsule, and loop back to drain into venules at the corticomedullary junction. These capillaries have tight intercellular junctions, which effectively prevent the exchange of macromolecular solutes between blood and tissue space, and which constitute the anatomical basis of the blood - thymus barrier (Raviola and Karnovsky, 1972). The medulla and CMJ, by contrast, have a more conventional blood supply of arterioles, capillaries, and postcapillary venules. The latter are particularly adapted to allowing cells to pass from the thymus to the circulation, and in this respect resemble the postcapillary venules of the lymph nodes.

Stutman and his collaborators (Stutman, 1977, 1978) have extensively studied the migration of stem cells to the thymus, and have given us our main insights into intra- and extrathymic events.

It was found that adult or embryonic stem cells could partially populate the thymus and subsequently the periphery of syngeneic unirradiated hosts, using as a source of stem cells, tissues from mice bearing the T6/T6 chromosome marker. T6/T6 cells were absent from the peripheral lymph nodes, but could be found in bone marrow and spleen of animals which had been neonatally thymectomized (nTx), or nTx and grafted

with thymus in a cell-impermeable diffusion chamber, indicating the necessity for an accessible thymic stroma for restoration of T cell function (Stutman and Good, 1971). Yolk sac cells were found to differ from foetal liver cells or bone marrow cells, in that they require an additional maturation step in the foetal liver during normal ontogeny, or in the bone marrow in the irradiated adult. Abrogation of marrow space function with ^{89}Sr resulted in failure of yolk sac cells to migrate to the thymus and periphery, while not affecting migration in animals given foetal liver cells or bone marrow (Stutman, 1977). Under these conditions, yolk sac-derived cells could be recovered from the spleen, and these cells could in turn repopulate the thymus and peripheral T cell pool of a subsequent host with a functional marrow space (Stutman, 1978).

The ability of chromosomally-marked stem cells to repopulate a non-irradiated host thymus was dependent on matching for histocompatibility antigens (Stutman and Good, 1969), including minor H, and this requirement could be overcome by supralethal irradiation of the thymus, suggesting that this was purely a competitive phenomenon, allogeneic stem cells being at a disadvantage in repopulating the graft (Stutman, 1978).

Emigration of cells from the graft is maximal in perinatal life, and subsequently declines (Bryant *et al*, 1975). In view of the substantial mitotic rate in the thymus, which is sufficient to allow complete replacement of peripheral T cells *de novo* five times per day, it has been postulated and shown that a majority of cells within the thymus have a brief lifespan of 3 - 4 days, and die within the thymic cortex. Shortman's elegant double-labelling experiments have shown that the proportion of short-lived cells in the thymus is at least 63% (Shortman, 1977; McPhee *et al*, 1979). Nonetheless, both short and long-lived cells are exported by the thymus, and Stutman has postulated that the former may represent 'post-thymic precursor' (PTP) cells, which may in turn give rise to long-lived peripheral cells.

Conventional wisdom (Weissman, 1973) holds that the medullary cortisone-resistant thymocytes represent the end-stage of thymic maturation, and are in fact fully mature cells awaiting export to the periphery. Studies by Weissman *et al* (1975), in which the thymic cortex was labelled *in situ*, showed that the medullary thymocytes appear to derive from the cortical population. Against this is the evidence of Claman and Moorhead (1972) that peripheral T cells contain both cortisone sensitive and

resistant populations, and the finding of Elliott *et al* (1971) that the intrathymic steroid-resistant population is apparently not subject to turnover, or to significant export to the periphery. Furthermore, this population cannot localize to the T cell dependent areas of the peripheral lymph nodes when transferred to irradiated hosts (Elliott, 1977). Stutman has subsequently shown that the majority of PTP cells exported by the thymus are steroid sensitive, but give rise to a population of steroid resistant cells in the periphery (Stutman, 1977).

The thymus secretes at least one humoural factor, thymopoietin, which is able to bring about the rapid expression of the Thy 1 alloantigen in spleen and bone marrow from congenitally athymic ('nude') mice *in vitro* (Komuro and Boyse, 1973). The peripheral tissues also contain mediators capable of rapidly inducing the expression of Thy 1 in 14 day foetal thymocytes, and which seem to act via the intracellular second messenger, cAMP (Singh and Owen, 1975, 1976). The initial enthusiasm over these findings, which suggested a potential role of these factors in the treatment of congenital or acquired immunodeficiency, was dampened by the subsequent demonstration that these cells, although bearing T cell markers, were not immunologically competent, and showed only modest responses to various T cell mitogens.

Stutman has carried out extensive studies on the restoration of T cell function in nTx mice, which shed light on this problem. Neonatally thymectomized animals could not be reconstituted beyond 40 days after birth with thymus grafts contained in cell-impermeable diffusion chambers. Immune competence could, however, be restored if thymus grafting was supplemented by the concurrent transfer of lymphocytes or stem cells from adult or neonatal animals, quite irrespective of the T cell content of these cells. Embryonic stem cells required the presence of a free (rather than an enclosed) thymus graft to allow reconstitution. These results were interpreted as showing the existence of two sorts of precursors of the T cell lineage; prethymic T cell precursors (predetermined T cell precursors) and postthymic T cell precursors (PTP), as discussed above. The prethymic precursors require maturation in the thymic stroma to become PTP, which are functionally immature precursor cells exported to the periphery (Stutman, 1978). It is these prethymic precursors which are present in nude mice, and which can be caused to display the antigenic phenotype of mature T cells (Thy 1$^+$, TL$^+$) *in vivo* (Stutman, 1974) or

in vitro (Basch and Goldstein, 1975), without a corresponding gain in function. Incubation of these cells with thymic hormone, or parenteral administration of the hormone, does not result in conversion to PTP, while incubation on thymic epithelial monolayers similarly fails to induce T cell function (Sato *et al*, 1976). The complexity of the necessary intrathymic differentiation is shown by the work of Robertson and Owen (1977), as *in vitro* cultures of thymic explants show rapid changes in antigenic composition, but require four days for the development of alloreactive cells. The above evidence thus suggests that in the absence of specialized contact with the thymic stroma, precursor T cells undergo a trivial pathway of T cell differentiation, without development of function.

The postthymic T cells, on the other hand, have been shown to give rise to competent T cells *in vivo*. PTP are in general characterized by sensitivity to steroids, adherence to nylon wool columns, and the expression of Lyt 1, and Lyt 2, 3 markers (Stutman and Shen, 1977); in short, the characteristics of much of the peripheral T cell pool.

PTP obtained in relatively pure form by velocity sedimentation will functionally reconstitute nTx mice simultaneously given diffusion chamber-enclosed thymus grafts, but not ungrafted nTx animals (Stutman, 1975). The most likely explanation for this is that thymic humoural factors mediate the maturation from PTP to functional T cells in the periphery. Functional T cells recovered from these animals, and responding to the T cell mitogen PHA, or to allogeneic T cells *in vitro*, were shown to belong to either the Lyt 1^+ or the Lyt 2, 3^+ subclasses, indicating the possibility that a common Lyt 1, 2, 3^+ precursor might give rise to both subclasses, with further differentiation occurring in the periphery.

Acquisition of T cell surface markers

The functional properties of cells provide a convenient, although purely empirical, means of assessing the state of maturation. Consequently, a number of studies have relied on the development of surface markers to explore the kinetics of ontogeny of the T cell system in foetal and neonatal animals, and in stem cell repopulated adults.

Owen and Raff (1970) carried out *in vitro* studies on the acquisition of the T cell markers, theta (Thy 1) (Reif and Allen, 1963), and TL, in foetal animals. Thymic rudiments were cultured for various periods of time on the chicken CAM, and the dissociated fragments analysed with cytotoxic antisera for their content of antigen-bearing cells.

Fourteen day foetal thymus was not found to express detectable theta alloantigen, as determined by direct cytotoxicity testing, but the antigen could be detected after two days in culture. The time of appearance of theta, and the proportion of theta-bearing cells was equivalent in cultured thymus and in normal thymus of equivalent gestational age. A concurrent histological maturation was observed during culture, with replacement of the relatively sparse large basophilic cells with large numbers of close-packed small to medium lymphocytes. The TL antigen was similarly absent from fourteen day thymus, but appeared after four days of *in vitro* culture. The proportion of cells killed by anti-TL antiserum was somewhat lower in cultured thymus than in normal eighteen day thymus. When the same cell population was analysed simultaneously for expression of theta and TL antigens, a similar discrepancy was found, suggesting the functional independence of the two antigen systems. Schlesinger and Hurwitz (1968) also analysed foetal thymuses for the presence of TL antigens, and found them to first appear at 15-1/2 days of gestation, with normal levels being attained by 16-1/2 days. Although this study gave results concordant with those of Owen and Raff, it cannot be conclusively shown that the alloantigen-bearing cells arose by differentiation from thymic precursors, and do not represent cells immigrating during the period of study. The technique of Owen and Raff overcomes this point, by cutting off the supply of immigrant cells at 14 days.

H-2 alloantigens are present on the stem cells infiltrating the thymic anlage at eleven or so days of gestation, and their expression reaches adult levels in late gestation, concurrently with the decline in surface theta antigen, and the loss of TL and linkage group IX markers.

Acquisition of T cell function

Perhaps of greater interest and relevance than the acquisition of surface markers is the development of functional immunocompetence; the ability to respond to mitogens and specific antigens. Analyses of this sort are technically difficult in the foetal mouse, owing to the small numbers of cells which can be recovered, 35000 - 50000 in the 14 day thymus. Mosier (1973, 1974), and Mosier and Cohen (1975) developed sufficiently sensitive *in vitro* microassays to allow them to examine the responses of thymocytes of 16 days of gestation or greater. Sixteen day foetal thymocytes responded well to PHA, and to allogeneic cells in the mixed lymphocyte reaction, but poorly to the mitogens

ConA and PWM. Neonatal cells, by contrast, showed enhanced ConA and PWM responses, attaining adult levels of reactivity by approximately three weeks after birth, while the PHA response decreased sharply at 19 days of gestation, and subsequently tended towards background levels. The allogeneic MLR showed a prenatal peak at 19 – 20 days of gestation, and then fell markedly at about the time of birth, although it subsequently recovered and reached adult levels by 2 – 3 weeks postnatally. The response to syngeneic spleen appeared at the same time as the response to allogeneic cells, and was greatest in the neonatal period. Reactivity gradually declined to background levels by two weeks after birth. The postnatal loss of reactivity to PHA and syngeneic or allogeneic cells is attributed by these authors to the development of a regulatory T cell subpopulation, which suppresses the response to 'self' permanently, and that to certain other antigens and mitogens at least transiently.

A somewhat different approach to essentially the same problem was taken by Robinson and Owen (1976, 1977), Juhlin and Alm (1976), Juhlin *et al* (1976, 1978), and Tufveson *et al* (1976). Fourteen day thymic rudiments were cultured *in vitro* for up to 28 days, and the functional capacities of the cells determined at various times after explantation. The responses of cultured thymus to various mitogens all appeared at approximately the same time; peaking at 11 days of culture for PHA, 12 days for ConA, and between 9 and 15 days for PWM. Cells from *in vivo* thymuses showed similar patterns of reactivity to those reported by Mosier (1974), with no detectable PHA response after birth, while PWM and ConA responses increased to adult levels within 2 – 3 weeks. The kinetics of acquisition of MLC responsiveness in cultured thymus were comparable to those obtained by Mosier with normal thymus, responsiveness appearing first at between 4 and 7 days in culture (18 to 21 days of gestation) *versus* 19 days of gestation for *in vivo* embryo thymus. The perinatal decline in response was not observed by either of these groups of authors, who reported that thymocytes from normal neonatal animals showed reactivity as great as those from adults; additionally, they did not observe reactivity of neonatal cells to syngeneic adult spleen.

The acquisition of T cell functions after birth has been rather more extensively studied, a reflection of the greater accessibility of this experimental system. Generally concordant results have been obtained by the different groups, chiefly Howe and

Manziello (1972), Stobo and Paul (1972), and Byrd *et al* (1973). Neonatal thymocytes are deficient in their ability to respond to PHA. Persistence of this deficit into adult life is claimed by all save Byrd *et al*, who reported that the PHA response increased and reached a plateau by 12 weeks after birth, although the response was subsequently obscured by rising background levels of proliferation. Conflicting results were obtained on the ability of neonatal cortisone-resistant thymocytes to be stimulated by PHA. Responses to ConA and PWM were relatively low in neonatal and early postnatal thymus, but attained full adult levels within a few weeks of birth. Similar results were obtained in NZB mice (Stobo, Talal and Paul, 1972).

The development of antigen-specific T helper cell function is difficult to assess, as it requires some form of an assay system, generally involving mature effector cells; B cells in the case of help for antibody production. Hence, a number of studies, of which those of Spear *et al* (1973) and Spear and Edelman (1974) are typical, showed that spleen cells from neonatal mice were immunologically incompetent for up to two weeks after birth, and that immune responsiveness subsequently appeared to thymus-dependent antigens, reaching adult levels by approximately eight weeks. Spear and Edelman showed that the kinetics of T and B cell maturation were different, mature B cells being present in neonatal spleen as judged by their ability to respond to a thymus-independent antigen (bacterial lipopolysaccharide), while the response to a thymus-dependent antigen (heterologous red blood cells) did not develop until later. As the proportions of cells bearing 'T' and 'B' surface markers did not change over the period of maturation of the response, it was proposed that T cells required post-natal maturation to become functionally immunocompetent.

Mosier and Johnson (1975) independently showed that B cells at a given stage in ontogeny were substantially more mature than T cells, in that adult spleen cells were able to provide help to neonatal spleen B cells in the *in vitro* response to sheep RBC. Neonatal spleen alone did not give an SRBC response. The number of adult spleen cells required to reconstitute the response of neonatal spleen B cells was substantially greater than that for adult spleen B cells, suggesting that neonatal spleen might contain a suppressive cell type. Treatment of the neonatal cells with anti Thy 1 and C' restored the response to the levels seen with adult spleen B cells. The authors interpreted these

results in terms of a Thy 1-bearing suppressor cell, capable of inhibiting exogenously-added helper cells, and also any helper cell activity present in the neonatal spleen. Further evidence in support of this conclusion came in the observation that neonatal thymocytes can suppress the *in vitro* responses to SRBC and TNP-Ficoll of adult spleen cells.

The development of *in vivo* responses to SRBC by neonatal cells was studied by Chiscon *et al* (1971), and Chiscon and Golub (1972). These authors used an *in vivo* adoptive transfer system in which various T cell sources were transferred into lethally-irradiated hosts, along with adult bone marrow as a (constant) B cell source. One or seven day neonatal thymocytes, but not 18 day foetal liver, were found to interact effectively with mature B cells in giving an antibody response to SRBC. T cell function in thymus reached adult levels by two days after birth, and could be detected in spleen by four days postnatally. In a later paper, Fidler *et al* (1972) showed that spleen cells of eight-day old mice cultured *in vitro*, or in a cell impermeable diffusion chamber *in vivo* failed to give an SRBC response. Additionally, they found neonatal mice to have mature adherent cell function in spleen, and that the immature cell population resided in the nonadherent fraction. Their interpretation of these results was based on the requirement for a humoural factor, or for some aspect of microenvironment, which was present in the irradiated host of the adoptive transfer model, but not otherwise. An equally plausible explanation, in light of the work of Mosier and Johnson (1975), is that the adoptive transfer system dilutes out the suppressive cell population in neonatal thymus, allowing expression of the helper cell function.

C. Ontogeny of B cells

In contrast to the situation just described for T cells, the ontogeny of B cells, at least in the mammals, is less well understood. The absence of a clearly-defined anatomical site for B cell development has meant that studies on B cell differentiation have had to rely heavily on the ability to display certain immunological functions as indicators of maturity, as well as surface marker studies.

B lymphocytes in Birds

In avian species, a strong association has been shown between the Bursa of Fabricius, a sac-like structure derived from an evagination of the dorsal wall of the cloaca at about the fifth day of embryonation, and the development of the B lymphocyte system (Cooper *et al*, 1966; Glick *et al*, 1956; Warner, 1964, 1965; Warner and Szenberg, 1962, 1964; and Weissman, 1975). The bursa is first populated by yolk sac haemopoietic stem cells at about 12 to 13 days of embryonation; later, spleen and bone marrow appear to serve as stem cell sources, as shown by the chromosome marker studies in parabiosed chick embryos of Moore and Owen (1966, 1967). Immunoglobulin M synthesis is initiated in the bursal follicles by day 14 of incubation (Kincade and Cooper, 1971); Cooper *et al* (1972) showed this to be principally cell surface or receptor Ig. IgG producing cells can be demonstrated in the bursa by the time of hatching.

Direct evidence for an important bursal role in the development of B cell immunocompetence has come from ablation studies, in which *in ovo* surgical (Cooper *et al*, 1969) or hormonal (Glick *et al*, 1956; Warner *et al*, 1969) bursectomy, or neonatal bursectomy with cyclophosphamide and testosterone (Lee and Paraskevas, 1972) results in a relative agammaglobulinaemia, and the failure to develop germinal centres and plasma cells in peripheral lymphoid organs.

Lerner *et al* (1972) and Bryant *et al* (1973) have reported, however, that hormonal bursectomy does not fully abrogate the antibody response, principally affecting primary IgM responses, and the high rate production of specific IgG antibody. Bursal agenesis was shown equally in antibody-producing and deficient hosts, leading Bryant to postulate a bursa-independent pathway of B cell differentiation in this situation.

A predominant bursal role in B cell development, though, is supported by the studies of Kincade *et al*, (1970), in which life-long agammaglobulinaemia was produced by *in ovo* treatment with anti-immunoglobulin antibody followed by surgical bursectomy at hatching, without affecting the maturation of the T cell system.

The migratory pathways of post-bursal cells have not been directly examined to any great extent, although isotope marker studies (Durkin *et al*, 1971) indicate that at least some cells enter the germinal centres of the spleen.

Mammalian B lymphocytes

The extensive evidence for a primary antigen-independent site of B cell differentiation in the chicken (*vide supra*), has led various groups, notably that of Good, to postulate a mammalian homologue of the bursa of Fabricius. Archer *et al* (1963, 1964), and Sutherland *et al* (1964), claimed the vermiform appendix of the rabbit to behave as a primary lymphoid organ, as its extirpation in neonates markedly reduced antibody formation. It was therefore postulated that the gut-associated lymphoepithelial tissues (GALT) and appendix might represent the bursa-equivalent (Cooper *et al*, 1966). Despite the considerable evidence which these authors adduce in support of their hypothesis (reviewed in Cooper and Lawton, 1973), other studies have failed to confirm this homology. Friedberg and Weissman (1974) have presented evidence from studies of the kinetics of cell proliferation in Peyer's patches which appears to exclude the GALT from even a minor role as a primary lymphoid organ in the mouse. Additionally, Weissman (1975) used immunofluorescence techniques to demonstrate that the distribution of functional cell types in Peyer's patches resembles that of secondary antigen-reactive tissues, rather than that of primary organs. Finally, Nossal and Pike (1973) did extensive autoradiographic surveys of foetal mice, which suggested a multifocal origin for B cells, beginning three days before birth, in liver, spleen, and bone marrow. These results, and those from other studies in which lethally-irradiated adult mice were repopulated with foetal liver cells, are apparently inconsistent with the existence of a single organ of primary B cell maturation.

Precursors of B cells in mammals are present in a number of foetal tissues, including the liver (Tyan and Herzenberg, 1968), and blood and yolk-sac (Stutman, 1973). The *in situ* generation of cells having B cell markers in foetal spleen and thymic explants was clearly shown by Owen *et al* (1974, 1975), with the *in vitro* culture system subsequently used by Robinson and Owen (1977) for studies on T cell ontogeny (*vide supra*).

Maturation of B cell Function

The first evidence that immunological competence to all antigens does not arise simultaneously came in the work of Silverstein *et al* (19[redacted] immunological competence of foetal sheep was assessed by their ability to mount immune responses to various antigens administered *in utero*. The earliest responses were obtained to ΦX 174, with a lesser response to horse ferritin. Ovalbumin was a weaker antigen, especially in young foetuses, while *Bacillus Calmette-Guerin*, diphtheria toxoid, and *Salmonella typhosa* were not immunogenic *in utero* or in early post-natal life. The inconvenience of working with sheep is obvious, and so it was with relief that studies turned to mice.

Detailed studies on the ontogeny of B lymphocyte function were first carried out by Chiscon and Golub (1972), using the same adoptive transfer protocol described above for T cell function. Briefly, B cell populations from various tissues were transferred into lethally-irradiated hosts, followed, after an appropriate interval, by adult thymocytes (as a source of mature T cells), and antigen (SRBC). B cell function, albeit at low levels (1/6 of normal adult bone marrow), was present in foetal liver 5 days before birth. Beginning at 60 hours before birth, foetal liver B cell function increased rapidly, and eventually surpassed that of adult bone marrow. With the decline in liver haempoietic function post-natally, functional B cells in this organ declined, and appeared in newborn spleen by four days after birth. These studies together constitute the first definitive evidence for nonsequential maturation of T and B cell function in the foetus and neonate.

Yung *et al* (1973) showed a dissociation between the ability of a cell to recognize antigen by binding, and to respond to it by the production of specific antibody. Lethally-irradiated adult animals were repopulated by the cells from single spleen colonies, each in turn the progeny of a single stem cell. The time course of development of antigen-binding cells and antibody-forming cells was determined for polymerized flagellin of *Salmonella adelaide*; ABC were detected at 18 - 20 days of lymphopoiesis, while AFC appeared later, at 19 - 26 days. Similar studies were performed for cells forming rosettes with SRBC (RFC), and making an anti-SRBC plaque-forming cell (PFC) response, with again the same dissociation; RFC appearing at 30 - 33 days, some 4 days before the first PFC.

The immunological properties of these antigens differ substantially with respect to their T cell dependence (Katz and Benacerraf, 1972), and thus the interpretation of these results is complicated by the failure to control for the maturation of T cell function occurring independently of B cell development.

This objection was largely overcome in the studies of Press and Klinman (1973), who used the splenic focus assay to detect and enumerate the precursors of AFC in various tissues. A constant source of mature T cells was provided by carrier-priming of the adoptive hosts; T cell function *in situ* being highly radioresistant (Katz *et al*, 1970). Limiting numbers of cells were transferred to the irradiated carrier-primed hosts, followed by removal of the seeded spleens, and *in vitro* culture of the spleen fragments in the presence of hapten-carrier conjugates. Antibody prouction by individual fragments was determined by radioimmunoassay. Each splenic focus presumably represents the clonal progeny of a single B cell precursor. Sixteen to nineteen day foetal liver, as well as spleen cells from animals in the first week after birth, can be stimulated to produce antibody on culture with DNP-KLH, and the frequency of anti-DNP precursors in neonatal spleen is not appreciably different from that in adults. Subsequent studies (Press and Klinman, 1974) further analysed the B cell precursor frequencies for the haptens DNP, TNP and fluorescein (FL) in neonatal and adult animals, reared under conventional or germ-free conditions. DNP and TNP precursor frequencies were virtually identical in neonatal and adult animals, irrespective of rearing conditions, while the frequency of precursors for FL was relatively lower in neonates, arguing in favour of differential rates of maturation for B cell precursors of different specificities. The equivalence of precursor frequencies in normal and gnotobiotic mice may be taken as an indication that generation of precursors is independent of stimulation by exogenous antigen.

Additional evidence for the early acquisition of B cell function (relative to T cell function) has come from the studies of Spear and Edelman (1974). I have also discussed the work of Mosier and Johnson (1975), suggesting high levels of suppressor T cell activity in neonatal spleen, which can be abrogated with anti-Thy 1 antibody, revealing substantial B cell activity.

The ontogenic hierarchy of responses suggested by the work of Yung *et al* (1973) and of Press and Klinman (1973) was subsequently verified by Sherwin and

Rowlands (1974), who used a series of eight antigens to look at the sequential development of immunity. Again the maturation of T cell function was not controlled for, so the hierarchical relationship to actual B cell maturation is difficult to assess.

Finally, Goidl and Siskind (1974) used the heterogeneity of affinity of the antibody response, as measured by hapten-inhibition, to determine the ontogenic maturity of B cell function. Heterogeneity of affinity was assumed to be a valid correlate of the polyclonality of the antibody response. Foetal liver cells were found to give a clonally restricted response of low affinity, which matured to an adult polyclonal pattern, with a substantial high affinity component, in the 1 - 2 weeks after birth.

A completely different approach to B cell ontogeny was taken by Lafleur *et al* (1972, 1973), who used sedimentation at unit *x* g to resolve between B cell differentiation states. With this technique, it proved possible to distinguish between pre-B cells, and B cells on the basis of size, although both types expressed surface Ig. The identity of the stem cells - perhaps equivalent to CFU-S - could not be defined, as they had a rather heterogeneous size distribution, although kinetic evidence for their existence was obtained.

As might be expected, newly differentiated B cells had physical properties closer to those of pre-B cells, than to mature B cells, although they were functionally equivalent to mature cells. Based on estimates of the physical size of AFC at various times, this pre-B to B cell transition was estimated to take 9 - 14 days.

D. Cellular interactions in the immune response

The unicellular concept of antigen recognition and antibody formation was first challenged directly in the work of Claman and colleagues (1966). These workers used an exceedingly simple *in vivo* adoptive transfer system to look for the presence of immunocompetent cells in adult thymus. Lethally-irradiated mice were injected with cells derived from spleen, bone marrow, or thymus, or a mixture of normal bone marrow cells and normal or immune thymocytes, syngeneic with the host animal. After SRBC challenge *in vivo*, PFC directed against SRBC targets were enumerated (Playfair *et al*, 1965).

Spleen cells were capable of giving a response on their own, while neither bone marrow cells nor immune or normal thymocytes alone gave rise to one. Mixtures of bone

marrow and thymus cells, by contrast, gave rise to a markedly synergistic response, related directly to the numbers of each cell type transferred. The authors concluded on this basis that bone marrow cells are able to generate antibody, but require the presence of thymus-derived auxilliary cells to do so.

Experiments leading to much the same conclusions were reported by Miller and Mitchell (1967). Bone marrow from neonatally-thymectomized mice was as effective as that from normal mice in restoring the SRBC response of lethally-irradiated normal animals. Allogeneic thoracic duct lymphocytes or thymocytes could restore the *in vivo* SRBC response in neonatally-thymectomized animals, and the spleen PFC obtained under these conditions could be blocked by anti-H-2 sera directed against the thymectomized host, rather than against the cell donor. These results clearly showed that the AFC is derived from the bone marrow, and is insensitive to the effects of neonatal thymectomy, although for effective antibody formation, the mediation of a thymus-derived cell, which also recognizes the antigen, is required.

Marbrook (1967) and Mishell and Dutton (1967) independently developed *in vitro* techniques for generating immune responses to particulate antigens, especially red blood cells, with dissociated spleen cell suspensions. Subsequently, Mosier *et al* (1968, 1970) employed similar techniques to directly identify the requirement for non-lymphoid accessory cells in the generation of an immune response. Initially, these authors demonstrated that mouse spleen cells could be separated into two functional subpopulations on the basis of adherence to glass surfaces. Neither adherent nor nonadherent cells alone could give a response, but showed marked synergy when combined in culture. It was found that macrophage-like cells were present in the adherent population, while most T and B cells were found in the nonadherent fraction. This conclusion was reinforced by studies with neonatally thymectomized mice. Spleen cells from these animals were incapable of giving a primary *in vitro* response, while the ability to respond could be restored by thymus-grafting of the thymectomized animals. The response of nonadherent cells from normal mice could be restored equally well by adherent cells from either normal or thymectomized mice, while nonadherent cells from neonatally thymectomized mice could not be made to respond by adherent cells from either normal or thymectomized animals. In this way, it was possible to localize the

immunological deficit in thymectomized animals to the nonadherent fraction, although the deficient cell type could not be clearly identified.

Subsequent work, in which T cells were depleted by treatment with anti-theta serum, or in which specifically primed T cells were used, clearly demonstrated that primary and secondary *in vitro* responses to erythrocyte antigens require collaboration between T and B cells (Chan *et al*, 1970; Hartmann, 1970, 1971; Munro and Hunter, 1970; Schimpl and Wecker, 1970, 1971).

It is relatively difficult, using erythrocyte or protein antigens, to establish the fine specificities of the antigenic determinants recognized by T and B cells. The elucidation of these points, both *in vivo* and *in vitro*, has relied heavily on the use of hapten-carrier conjugates.

In the years preceding the formulation of the concept of hapten and carrier, certain phenomena had been described which can (retrospectively) be explained in these terms. For example, immunization with hapten was known to elicit anti-hapten antibodies only when the hapten is conjugated to an immunogenic carrier; poorly immunogenic substances are likewise poor carriers (Levine *et al*, 1963). Ovary and Benacerraf (1963) also described a 'carrier effect', namely that for optimal secondary antibody responses to hapten, the priming and recall carriers must be the same. Given that anti-hapten antibodies react minimally with the carrier, this argues in favour of a distinct carrier recognitive step, a point which subsequent work has amply verified.

Immune response or *Ir* genes similarly provided clear evidence for a hapten-carrier effect. Rajewsky *et al* (1967) studied the development of antibodies to the tetrameric LDH isoenzyme in rabbits. Certain rabbits were found to respond to both the I and V subunits with antibody production, while others responded to V. The latter animals, on rechallenge with a I-V hybrid tetramer, gave a response to both I and V, suggesting that the presence on the same molecule of the V monomer in some way facilitated the response to the normally nonimmunogenic I subunit.

The first direct evidence for separate recognition of hapten and carrier specificities by independent cell types in the *in vivo* antibody response was obtained by Mitchison (1969). He showed that NIP-OVA primed spleen cells, transferred into syngeneic, irradiated (600 rad) mice, gave a secondary anti-NIP response on rechallenge

with NIP-OVA, but not to the heterologous conjugate, NIP-BSA. A good secondary response to NIP-BSA was obtained, however, when BSA-primed spleen cells were injected along with those primed to NIP-OVA. Thus, addition of cells specific for the heterologous carrier allows a perfectly good response to NIP-BSA by NIP-OVA primed (B) cells.

The analogy between hapten and carrier, and bone marrow and thymus synergy is striking, and Raff (1970) conclusively confirmed this, in his now-classic demonstration that (carrier-specific) helper cells are thymus-derived, while the (hapten-specific) AFC, are not. Hapten-carrier primed spleen cells were transferred to syngeneic irradiated mice, and challenged with homologous hapten-carrier conjugate; a normal PFC response was obtained at seven days after transfer. The same spleen cells challenged *in vivo* with a heterologous hapten-carrier failed to respond, while anti-theta treatment of the primed spleen cells abrogated the response to either homologous or heterologous hapten carrier conjugates. Anti-theta treated, primed spleen cells, when mixed *in vivo* with spleen cells primed to the heterologous carrier, gave a response only to the heterologous carrier-hapten conjugate. This last response could be abrogated by treatment of the carrier-primed spleen cell population with anti-theta serum, clearly showing the helper cells to have T cell markers.

Rajewsky *et al* (1969) and Katz *et al* (1970) independently arrived at a similar conclusion, although with an altogether different approach. Rather than using the adoptive transfer of heterologous carrier primed T lymphocytes to circumvent the normal carrier effect, these authors primed animals with hapten-carrier conjugates, and then gave a supplemental immunization with a heterologous carrier. Secondary *in vivo* immune responses could subsequently be elicited by challenge with either the homologous or the heterologous carrier-hapten conjugate.

E. Major histocompatibility complex control of immune responses

Genes encoded by the major histocompatibility complex are known to control the quality and degree of immune responses to various antigens. It seems likely that the overall immune response to a complex or multideterminant antigen represents the sum of the immune responses to the individual determinants; hence much of our understanding

of these phenomena has come from the study of simple antigens, in which at most a few determinants are involved in eliciting a response.

Several classes of molecules have been shown to have these properties, including synthetic peptide antigens of either random or defined structures; alloantigens differing only slightly from their autologous counterparts; and complex antigens administered in very low doses such that only the most immunogenic determinants are recognized. Most attention recently has focussed on the responses to the first two classes of antigens, yet our understanding of immune response (*Ir*) gene action owes much to the last approach. Hence I will deal with the latter antigens first, and with the others subsequently.

History of immune response genetics

The first evidence for an association between the genotype and the ability to mount an immune response derives from the work of Gorer and Schutze (1938), on *Salmonella* infection in genetically-resistant mouse strains.

Schiebel (1943) obtained direct evidence for a genetic influence on antibody formation, with his elegant breeding studies in the guinea pig. Random-bred guinea pigs were immunized with diphtheria toxoid, and assigned to 'high' and 'low' responding groups. Selective inbreeding of high responders, and of low responders, led over several generations to the establishment of populations with uniformly high or low responsiveness. As these stable populations were derived within a few generations of inbreeding, it appeared that relatively few genes were involved in determining responsiveness.

Twenty-five years later, Biozzi *et al* (1968) used a similar approach to select for responsiveness to sheep red blood cells in outbred mouse populations; interestingly, the agglutinin response to pigeon red blood cells segregated with that to SRBC.

The availability of large numbers of inbred mouse strains beginning in the 1950's led to the description of reproducible inter-strain variability in responses to a battery of antigens, first by Fink and Quinn (1953), and subsequently by Ipsen (1959), Dineen (1964), and Playfair (1968). von Sengbush and Lennox (1969) analysed the responses of some 20 inbred mouse strains to influenza viruses, finding both high and low responders. In general, high responsiveness was acquired as a dominant trait, (HR x LR)F_1 crosses being HR; while evidence for complementation of multiple genes was found in the mating of

two particular low responder strains, the F_1 progeny being high responders.

These studies are chiefly of historical interest, in that they show certain properties of *Ir* genes, *viz.* the dominance of responsiveness, multigene control (in some instances), and Mendelian patterns of segregation. The techniques and antigens used were altogether inadequate, however, to allow the elucidation of the molecular and cellular mechanisms of *Ir* gene action. Subsequent studies have relied exclusively on antigens of defined structure, or of limited heterogeneity.

Poly-L-Lysine

Until recently, the only antigen in the first category has been the linear homopolymer, poly-L-lysine (PLL), and it is still prototypical in many respects. Kantor, Ojeda, and Benacerraf (1963) observed that approximately 30% of random-bred Hartley guinea pigs respond to DNP-PLL by the development of DTH, and the production of large amounts of serum antibody directed against haptenic and carrier determinants. Lymph node cells from responding animals give *in vitro* proliferative responses to the antigen (Green *et al*, 1968), while nonresponders display none of these parameters of immunity. Conveniently, the two available inbred strains of guinea pigs differ in responsiveness to DNP-PLL, Strain 2 responding with DTH and serum antibody, and Strain 13 showing no responses (Levine *et al*, 1963a). Responsiveness is clearly a function of the carrier, as unconjugated PLL (Green *et al*, 1966), and PLL conjugated to other haptens (Levine *et al*, 1963b), give similar patterns of response as DNP-PLL.

The responses to the random copolymer $Glu^{60}Lys^{40}$ (GL), and DNP-GL are under the same genetic control as the PLL response, despite the lack of cross-reactivity between the two antigens (Kantor *et al*, 1963), arguing in favour of a single immune response gene, or a family of closely-linked genes.

The genetic control of the PLL and GL responses was analysed in extensive mating studies with random-bred Hartley guinea pigs, and in inbred Strains 2 and 13 (Benacerraf *et al*, 1967). The ability to respond to both of these antigens, and their hapten conjugates, is inherited as a single autosomal dominant, termed the PLL gene.

Schlossman *et al* (1965, 1966, 1967) established that the minimum immunogenic size PLL peptide is the octalysine. This minimum applies both to priming, and to the elicitation of anamnestic responses *in vivo* or *in vitro* (Stulberg and Schlossman, 1968).

The PLL molecule is highly charged under conditions of physiological pH and ionic strength, and thus can form stable aggregates with various foreign albumins. Provided the albumin is itself immunogenic, it is able to act as a carrier for DNP-PLL, and elicit antibody responses in PLL-nonresponder animals. In this situation, DNP-PLL acts purely as a hapten, and the usual concomitant to antibody, the development of DTH, is not seen (Green *et al*, 1966, 1968; Theis *et al*, 1969).

The PLL molecule suffers from the distinct disadvantage of being nonimmunogenic in the mouse (Pinchuk and Maurer, 1965), severely restricting its usefulness in genetic studies. At about the same time as the studies with PLL were initiated, Arquilla and Finn (1963) began to study the genetic control of the immune response to purified insulins, showing that strain 2 and strain 13 guinea pigs produce antibodies directed against different portions of the same molecule. Chemical modification of the insulin molecules established that strain 2 guinea pig antibodies react preferentially with the N-terminus of the insulin A and B chains, while strain 13 anti-insulin antibodies react with the C-terminal end (Arquilla *et al*, 1967). These studies of naturally-occurring proteins and synthetic peptides have since given rise to exceedingly profitable studies into the nature of the antigenic determinant recognized by the T cell, and of the mechanisms of *Ir* gene action.

Random amino acid copolymers

The studies of Maurer and Pinchuk (1968) established that the immunogenicity of random amino acid copolymers increases with increasing complexity of the molecule (terpolymers are more immunogenic than copolymers, which are in turn more immunogenic than homopolymers). Indeed, the addition of a few mol % of a third amino acid to the nonimmunogenic copolymers GT, GA, or GL allowed the development of a good immune response in most strains of animals tested. The GLA^5 (ie. 5 mol % A) terpolymer, however, revealed a clear pattern of responder and nonresponder strains (Pinchuk and Maurer, 1965). The antibodies elicited by this and related antigens in responder strains are highly cross-reactive, in that they can be absorbed by simpler copolymers, such as GL or GA. Thus, the capacity of an animal to recognize specific determinants, rather than its ability to produce a given idiotype, is genetically-controlled.

Direct evidence for a single genetic locus controlling the immune response to a synthetic peptide antigen was obtained by McDevitt and Sela (1965), with the branched chain polypeptides (T,G)-A--L and (H,G)-A--L. The A--L backbone of the molecule is nonimmunogenic (see Pinchuk and Maurer, 1968), and immunogenicity is conferred by short random sequences of (T,G) or (H,G) attached to the poly-D,L-lysine side-chains. CBA mice respond well to (H,G)-A--L, and poorly to (T,G)-A--L, while the reciprocal pattern of response is seen in C57 mice. The ability to respond was found to be a genetically determined quantitative trait, controlled by an autosomal dominant gene, denoted Ir-1 (McDevitt and Sela, 1965, 1967; McDevitt, 1968). McDevitt (1968) showed that Ir-1 is not linked to the Ig region coding for mouse heavy chain immunoglobulin allotypes, and an association between H-2 and Ir-1 was first suspected on the basis that C3H.SW (H-2*b*) but not C3H.DiSn (H-2*k*) mice responded to (T,G)-A--L, these strains being identical at all loci except H-2. Similar results were obtained with C57BL/10 (H-2*b*) and B10.BR (H-2*k*) mice, and the linkage was confirmed in backcross tests, in which (T,G)-A--L responsiveness segregated with the H-2*b* allele (McDevitt and Tyan, 1968). McDevitt *et al* (1969) used a series of recombinant strains between responders and nonresponders to map the Ir-1 locus within H-2, and McDevitt and Benacerraf (1969) proposed that Ir-1 might code for then-undetected H-2 specificities expressed on the cell surface, which control recognition of specific antigens.

The independence of H-2 linked specific immune responsiveness from antibody structural genes was shown indirectly by Bechtol and Press (1976), Benacerraf and McDevitt (1972), and Dorf *et al* (1975). All of these authors showed striking cross-reactivities between antibodies directed against related synthetic peptide antigens, and yet quite distinct distributions of responders and nonresponder strains for the various antigens. These findings indicate that the H-2 linked *Ir* genes do not directly determine the antibody specificities expressed.

The first evidence for non-H-2 linked *Ir* genes was obtained by Mozes *et al* (1967a, b), with the peptides (T,G)-Pro--L, and (Phe,G)-Pro--L. Responsiveness to these antigens was localized to the Ir-3 locus.

Immune response genes linked to immunoglobulin allotypes have been identified by a number of workers including Eichmann (1972), Blomgren *et al* (1972), and Pawlak

and Nisonoff (1973). These *Ir* genes control the fine specificities of antibodies elicited by immunization with certain antigens, for example, bacterial carbohydrates. They are not responsible for overall variations in the amounts of antibody produced, but instead regulate the expression of particular idiotypes. As immunoglobulin allotype markers are associated with the heavy chain constant region , and the constant and variable regions are known to be linked (Landucci-Tosi *et al*, 1970), it is thought that these allotype-associated *Ir* genes in fact code for the variable regions of the antibody molecules.

Both H-2 linked and allotype linked *Ir* genes must in some way be related to specific recognition of antigens. The clear dissociation between the two classes of genes indicates, however, that H-2 linked recognition must be independent of immunoglobulin idiotype, and thus requires that an alternative means for specific recognition of antigen exist. In light of these considerations, Benacerraf and McDevitt (1972) suggested the existence of a functionally and structurally distinct T cell receptor for antigen. In view of the close functional association between MHC products, and H-2 linked *Ir* genes (Hammerling and McDevitt, 1974; Shevach *et al*, 1972, 1974), this receptor was proposed to express MHC-encoded determinants, as well as specificity for antigen (Katz and Benacerraf, 1975).

Cellular expression of immune response genes for synthetic peptide antigens

Extensive studies at the cellular level have been carried out on the immune response genes associated with the PLL locus in the guinea pig, and with the Ir-1 locus in the mouse. The studies of Foerster *et al* (1969) and Ellman *et al* (1970) established that responsiveness to antigens controlled by PLL could be transferred to nonresponder animals with lymph node and spleen cells from responders, and that the afferent arm of the response was entirely dependent on the transferred donor cells. These studies did not directly address the question of *Ir* gene expression in the efferent arm; nonetheless, the data suggest that they are expressed in a lymphoid, rather than a macrophage/monocyte, cell type.

Evidence that *Ir* genes are expressed in T cells comes from a number of studies, and includes that all *Ir*-controlled antigens are thymus-dependent, and that *Ir* gene effects can be shown not only for antibody but also for cell-mediated immune functions.

H-2 linked *Ir* genes are involved in recognition of carrier rather than haptenic determinants, carrier recognition being a T cell function. In an extension of this last point, responses to *Ir* gene controlled hapten-carrier conjugates can be elicited in strains lacking the necessary *Ir* gene, by complexing the antigen with an immunogenic carrier (Green *et al*, 1969). The conjugate here acts as a hapten, and elicits an antibody response indistinguishable from that given by responder animals; implying that nonresponder B cells are not deficient in their ability to give a response to determinants on *Ir*-controlled antigens. Additionally, antigen-binding cells (ABC) are found at comparable frequencies in responder and nonresponder animals (Dunham *et al*, 1972).

Nonetheless, substantial evidence has accumulated to suggest that other levels of *Ir* gene control than at the T cell may exist. This evidence takes two forms; evidence against *Ir* gene expression by the T cell, and evidence for other sites of expression.

In the former category come a number of observations by Kapp and collaborators, on the generation of *in vitro* or *in vivo* immune responses to the terpolymer GAT. Thus, administration of GAT to nonresponder strains of mice apparently fails to generate helper T cells, and also gives rise to a population of suppressor cells (Kapp *et al*, 1974), whose activity can be shown *in vivo*, by a reduction in the response to a subsequent challenge with a normally immunogenic dose of GAT-MBSA. These suppressor cells also act *in vitro* on normal spleen cells in the response to GAT-MBSA. Antigen-binding T cells have been shown at comparable frequencies in both high and low responder strains, with two different antigen systems (Hammerling and McDevitt, 1974b; Kennedy *et al*, 1975). This finding argues against *Ir* genes and the putative T cell antigen receptor having a structural relationship (*vide supra*). Additionally, GAT-specific T helper cells can be elicited in nonresponder animals by priming with GAT or GAT-MBSA-pulsed syngeneic macrophages. This help can, however, be shown only *in vitro*, and only with semiallogeneic B cells. Syngeneic B cells are not activated by these 'nonresponder' T cells, but can be stimulated by primed semiallogeneic 'responder' T cells, perhaps indicating that the balance between helper and suppressive effects is mediated by events occurring distal to antigen recognition (Kapp *et al*, 1975). This result is compatible with a predominant regulatory role for *Ir* genes, and suggests that these may be expressed at either the T or B cell levels.

The importance of strain differences in assessing the action of H-2 linked genes is emphasized in the studies of Marrack and Kappler (1979) with (T,G)-A--L, an antigen for which *Ir* gene expression has been claimed at the macrophage and B cell levels, in animals of H-2*a* and H-2*f* haplotypes.

T cells from LR -> (HR x LR) chimaeras were primed *in situ* in the context of antigen on HR antigen-presenting cells (APC); helper cells of H-2*a* haplotype cooperated with APC and B cells of the appropriate HR haplotype *in vitro*, while those of the unrelated H-2*f* haplotype did not. This result suggests the expression of *Ir* genes for (T,G)-A--L by helper T cells, as well as by APC and B cells, in animals of H-2*f* haplotype, but only by APC and B cells in H-2*a* strains. This result is easier to reconcile with a functional role for *Ir* genes in T cells; for example, the induction of suppressor T cells, than with a structural role. The latter implies the absence of functional T cell receptors for (T,G)-A--L in H-2*f* mice, a point which seems unlikely.

The entire question of *Ir* gene expression by B cells is particularly murky. Shearer *et al* (1972) claimed to find deficits in both bone marrow and thymocyte populations, when limited numbers of cells were used to reconstitute the *in vivo* response to (Phe,G)-A--L in SJL mice. Kapp *et al* (1974), again with the GAT system, showed that (R x NR)F_1 T cells could provide help only to responder B cells in the presence of soluble GAT, but to both responder and nonresponder B cells, in the presence of GAT-MBSA or GAT-pulsed macrophages, a result compatible with *Ir* gene expression by the B cell. Additionally, nonresponder B cells could readily be tolerized by doses of soluble GAT normally highly immunogenic for responder cells. Benacerraf *et al* (1974), however, in a companion paper, presented evidence which, in retrospect, makes B cell expression of *Ir* genes less likely, and which is more consistent with T - B cell restriction, and *Ir* gene controlled activation of an H-2 restricted helper T cell subpopulation.

Marrrack and Kappler have over the last few years carried out extensive studies on *Ir* gene expression by various of the cells involved in the immune response. In addition to the evidence for *Ir* genes acting on helper T cell function in at least one haplotype (*vide supra*), they have claimed to find *Ir* gene expression at the macrophage and/or B cell levels, using a modification of the Kapp *et al* (1974) protocol for GAT.

An *Ir* gene was described as controlling the extent of BRBC/SRBC cross-reactivity (XR) at the helper T cell level, and was mapped to between K and I-E by the use of H-2 recombinants. In initial studies, primed helper T cells from (HXR x LXR)F_1 animals were assayed with HXR or LXR macrophages and B cells, and were found to cooperate only with cells of HXR haplotype. When the same helper T cells were assayed with MØ of HXR haplotype, and various B cells, a response was obtained only with HXR B cells, localizing *Ir* gene expression to the B cell, and certainly not excluding the APC (Kappler and Marrack, 1977).

More detailed studies were subsequently done with the *Ir* controlled antigen, (T,G)-A--L. The ability of *in situ* primed (HR x LR)F_1 T cells to cooperate with HR or LR B cells and APC was assessed, and restriction to HR haplotype was again found; low responder B cells of H-2*h2*, H-2*k*, and H-2*f* failed to respond in the presence of F_1 T cells and APC. This result ascribes at least one site of *Ir* gene expression in these strains to the B cell. Evidence for APC (as well as B cell) defects in H-2*a* mice was obtained when primed F_1 helper cells were added to HR or LR B cells, along with antigen-pulsed HR or LR APC. LR B cells failed to respond to antigen on APC of either type, while HR B cells cooperated only with HR APC (Marrack and Kappler, 1978). Finally, cells from P -> F_1 and F_1 -> P bone marrow chimaeras were used to investigate the roles of T cell genotype and host environment on the expression of *Ir* gene controlled functions. Cells from P -> F_1 chimaeras cooperated in an unrestricted fashion with B cells and APC from either of the parents in the F_1, in the response to non-*Ir* controlled antigens,such as SRBC. However, in the (T,G)-A--L response, LR -> (HR x LR)F_1 T cells cooperated only with HR B cells and APC; while F_1 -> HR but not F_1 -> LR, T cells cooperated with HR APC and B cells. Irrespective of the T cell source, LR APC and B cells did not respond to (T,G)-A--L under any conditions. The authors interpret these results as consistent with the expression of *Ir* genes by the APC and/or B cell in the response to (T,G)-A--L. Additionally, they claim the T cell genotype not to influence the ability of the cells to be primed, but, rather, that the genotype of the chimeric host is crucial in this. Thus, LR T cells differentiating in an (HR x LR)F_1 environment, but not (HR x LR) cells in an LR host, can be primed to cooperate with HR B cells and APC. This suggests that T cell function is affected by *Ir* genes during differentiation, but that mature T cells do not express

functional *Ir* genes, particularly as these may not be encoded in the germ-line.

All of the above studies claiming to show *Ir* gene expression by the B cell have a common conceptual problem, namely that they require that *Ir* genes be non-clonally expressed on B cells; or, in other words, that all B cells must have all *Ir* genes. It is relatively simpler to postulate clonal expression of *Ir* genes on T cells, and alternative explanations for most of the above results can be proposed; probably the simplest is based on the work of Swierkosz *et al* (1978). These authors showed normal F_1 T cells to comprise two distinct subpopulations, each restricted to one of the parental H-2 haplotypes. Sprent and von Boehmer (1979) arrived at a similar finding for P -> F 1 chimaeric cells. Seen in these terms, the inability of primed (HR x LR)F_1 T cells to cooperate with LR B cells simply represents a failure to prime the LR-restricted subpopulation of F_1 T cells, a result quite compatible with *Ir* gene expression at either the T cell or the APC. The chimaera results can be explained similarly, although they are more consistent with expression at the APC level.

Expression of immune response genes by the antigen-presenting cell has been and still remains a point of controversy, although there is now good evidence for it. Kapp *et al* (1973) investigated the cellular requirements for the induction of primary *in vitro* antibody responses to GAT and GAT-MBSA. APC were necessary for the development of a response to either of these antigens in responder strains, and to GAT-MBSA in nonresponders. Nonresponder APC could present GAT or GAT-MBSA to responder nonadherent cells, arguing against an APC deficit in nonresponders, while R APC supprted the responses of NR nonadherent cells to GAT-MBSA, but not to GAT, indicating the absence of GAT-specific helper cell function in NR. Subsequently, Pierce *et al* (1976) claimed to be able to prime GAT-responder cells *in vivo* with GAT-pulsed NR APC, and to elicit *in vitro* secondary responses with R or NR GAT-pulsed APC., a result consistent with *Ir* gene expression at a level other than the APC. However, allogeneic effects could account for at least some of these observations, which are inconsistent with our current understanding of H-2 restriction.

In a quite different system, *in vitro* T cell proliferation in response to antigens under the control of the PLL gene in guinea pigs, Shevach and Rosenthal (1973) claimed to find evidence for expression of *Ir* genes by antigen presenting cells. Primed

(R x NR)F_1 lymph node cells proliferated in response to antigen presented on responder, but not nonresponder, APC. Together with their earlier paper (Rosenthal and Shevach, 1973) showing that F_1 LNC could be stimulated by non-PLL controlled antigens associated with APC of either haplotype, this suggests a selective deficit in APC function in nonresponders. The failure of NR APC to stimulate F_1 cells on rechallenge *in vitro* may reflect an inability of NR APC to prime responder T cells *in vivo*; equally, it may indicate that the appropriate subset of F_1 T cells cannot be activated under these conditions, which would imply a T cell deficit.

Yano *et al* (1978) obtained comparable results with the terpolymer, GAT, in the mouse. It was determined that T cells and APC must have in common the high responder allele at the I-A subregion of H-2, for the induction of a proliferative response. Further evidence for *Ir* gene expression by the APC was provided by Singer *et al* (1978). The *in vitro* response to (H,G)-A--L or (T,G)-A--L of (R x NR)F_1 nonadherent spleen cells was restored by F_1 or R spleen adherent cells, but not by NR cells; while any of the populations of spleen adherent cells would reconstitute the response to a non-*Ir* controlled antigen. These studies did not, however, address the question of *Ir* gene function at the T or B cell levels, nor did it exclude the possibility of expression at more than one level, as has been reported by Marrack and Kappler (1978).

Hodes *et al* (1979), again using the TNP-(T,G)-A--L system, localized *Ir* gene expression to only the APC, and not T or B cells in H-2*a* (low responder) animals (cf. Marrack and Kapppler, 1979). Spleen cells from LR -> (HR x LR)F_1 chimeras were found to be able to help either HR -> F_1 or LR -> F_1 B cells in the presence of HR but not LR spleen adherent cells. Similarly, only HR SAC could reconstitute the TNP-(T,G)-A--L response of (HR x LR)F_1 nonadherent spleen cells *in vitro*. A possible reason for this discrepancy is that Hodes *et al* used TNP-primed B cells in their assay system, while Marrack and Kappler used B cells activated by TNP-LPS. Thymus-dependent antigens and TNP-LPS are known to activate different B cell subpopulations, and functional expression of *Ir* genes may be quite different under these conditions. Alternatively, apparent *Ir* gene function in B cells may simply reflect the expression of *Ir* genes by residual APC in the cell preparations.

Erb *et al* (1980) used allophenic and irradiation chimaeras to look for expression of *Ir* genes at the T cell and APC levels for a series of *Ir*-controlled antigens. It was found that LR stem cells differentiating in an (LR x HR)F_1 environment, or in a LR <-> HR tetraparental could acquire the HR phenotype. Antigen-presenting cells of LR genotype did not, however, undergo modulation to HR phenotype under these conditions. These results permit at least two interpretations; expression of *Ir* genes by the T cell, or expression by the host and APC. The first interpretation would require the absence of functional T cell receptors for the antigen, a point for which there is no evidence. More attractive is the possibility that both the host and the APC must express the identical *Ir* genotype for responsiveness, and that defects at either site lead to nonresponsiveness. Thus, the host would have a permissive role, selecting for T cell subsets with the capacity to recognize particular Ia antigens, while the APC functions in determinant selection. As suggested by the work of Zinkernagel (1978c), the site of host selection may be the thymus.

In separate experiments, Hedrick and Watson (1979) showed the influence of thymic haplotype in determining responsiveness to the *Ir* gene controlled antigen, calf skin collagen. (HR x LR)F_1 ATxBM or nude mice were grafted with HR or LR thymuses, and animals showing restoration of the SRBC response were immunized with collagen. The collagen response was clearly determined by the responder or nonresponder status of the thymus, indicating that the overall immune response phenotype of an animal is determined by H-2 antigens expressed by the thymus during ontogeny, when the influence of *Ir* gene expression by the APC is eliminated.

Immune response gene complementation

The unigenic concept of MHC-linked immune response gene action was first challenged in the studies of Merryman and Maurer (1975) and Dorf *et al* (1974). These groups identified distinct *Ir* genes for the GL6 synthetic peptide, mapping to I-C and I-A respectively. The I-C associated gene is termed beta, and the other alpha, a definition based on the ability of the alpha and beta genes to complement one another, but not alpha with alpha (beta with beta; see below). Thus strains possessing responder alpha and beta alleles (alpha+, beta+) are responders, while (alpha-, beta+) or (alpha+, beta-) strains are nonresponders. An F_1 hybrid between the latter two strains is a responder, indicating

that the genes can function in either *cis* or *trans* positions. Evidence for (beta, beta) complementation in the absence of functional alpha genes is obtained in (B6 x SJL)F_1 hybrids (Decf and Benacerraf, 1975).

The cellular expression of these *Ir*-GLó genes has been extensively studied, beginning with Schwartz *et al* (1976), and Katz *et al* (1976). The former used an *in vitro* T cell proliferation assay to show that peritoneal exudate T cells had to express responder alleles of both the alpha and beta genes simultaneously to give a response. This result is consistent with *Ir* gene expression by T cells. These studies did not, however, address the question of other levels of *Ir* gene expression, for example, at the APC or B cell. Katz *et al* (1976) obtained evidence suggesting that T and B cells must both express the (alpha+, beta+) phenotype simultaneously for cooperation, with an *in vivo* adoptive transfer system for antibody, and, further, that alpha and beta responder alleles should be in *cis* position for optimal cooperation. A similar requirement for expression of (alpha+, beta+) phenotype by both APC and T cells was shown by Schwartz *et al* (1978) using the T cell proliferation assay. (B10.A x B10)(alpha+, beta−) x (alpha−, beta+)F_1 hybrid T cells could be stimulated by GLó-pulsed F_1 (alpha, beta *trans*) or B10.A(5R) (alpha, beta *cis*) APC, but not by B10, B10.A, or (B10 + B10.A) APC. This is consistent with the notion that responder immune function to GLó requires the codominant expression of the alpha and beta alleles of the *Ir*-GLó gene complex in at least some of T cells, B cells, or APC.

Further evidence for this conclusion comes in the work of Schwartz *et al* (1979). GLó-primed T lymphocytes from [(alpha+, beta−) x (alpha−, beta+)]F_1 responder animals were stimulated with antigen-pulsed nonimmune spleen cells (as a source of functional APC). Only responder complementing F_1 and recombinant cells can present GLó; neither of the complementing cell populations, alone or together, will do so, although they can present PPD. Thus, the APC is at least one cell type in which *Ir*-GLó genes are expressed.

Chimaeras of various types have also been used to dissect the cellular mechanisms of complementing *Ir* gene action. Warner *et al* (1977) constructed tetraparental mice by fusion of embryos of strains C57BL/6 and A/J, which show gene complementation in the F_1 in the response to GLó. None of the mice produced antibody

on immunization with GLó *in vivo*, not surprising in light of the requirement for expression of both *Ir*-GLó genes in the APC. Unfortunately, this negates the authors' claim to show a lack of complementation between alpha and beta genes expressed in separate T cells. This latter point was also approached by Schwartz *et al* (1979), with radiation chimaeras [(alpha+, beta−) + (alpha−, beta+) -> (alpha+, beta− x alpha−, beta+)F_1]. Despite that the F_1 host is a high responder, LR parental-derived cells are unable to respond to GLó, arguing that both complementing *Ir* genes must be expressed in the same cell. This system also suffers from the same problem discussed above, *viz*, inability to prime *in situ* with high responder APC, and thus to look directly at the T cell repertoire.

Longo and Schwartz (1980) finally overcame this difficulty, by priming T cells from complementing *A* + *B* -> (*A* x *B*)F_1 bone marrow chimaeras *in vivo* in acutely-irradiated (*A* x *B*)F_1 animals, with F_1 bone marrow providing F_1 (responder) APC. Under these conditions, T cells of either parent proliferated to GLó, showing that both complementing *Ir* genes need not be present in the same T cell. Collaboration between the two parental T cell populations, resulting in effective complementation (a two T cell model), was excluded on the basis that (F_1 -> *A*) and (F_1 -> *B*) T cells did not complement in the GLó response. Finally, it was shown that the *Ir* phenotype of T cells can be fully acquired, as stem cells from an animal of (alpha−, beta−) genotype differentiating in a complementing [(alpha+, beta−) x (alpha−, beta+)]F_1 host are able to give a proliferative response to GLó when primed with F_1 APC (Longo and Schwartz, 1981).

Immune response genes and antigens of known structure

Until comparatively recently, studies on *Ir* gene control of immune responses have relied heavily on the use of random synthetic polymers of amino acids. Although these antigens have been exceedingly useful for this purpose, they display a heterogeneity of molecular weight and spatial configuration, and may have different antigenic properties depending on the preparation used. Additionally, even a single molecule must possess a substantial range of antigenic determinants. Two major approaches may be taken to overcome these problems; the synthesis of polypeptide antigens of known primary sequence and thus controlled secondary and tertiary configurations, or the use of naturally-occurring genetic variants of proteins generally

well conserved in phylogeny.

The first is relatively little used as yet, although Singh and his colleagues have described the synthesis (Barton *et al*, 1977) and immunological properties of defined sequence peptides (Singh *et al*, 1978). Initial studies were conducted with the octadecapeptide TNP-18, or [Glu-Tyr-Lys(TNP)-(Glu-Tyr-Ala)$_6$]n, synthesized by fragment condensation. This antigen has an alpha-helical configuration, with a linear spacing of 27 Angstroms between adjacent haptenic groups. The ability to respond to this antigen has been shown to be associated with the H-2*d* haplotype in mice, and to map to between K and I-E by the use of the C3H.OH and C3H.A recombinant strains. Responsiveness was inherited in (R x NR)F$_1$ animals as a dominant trait. Unlike the situation with GAT (Kapp *et al*, 1974), but similar to that seen for insulin by Keck (1975a), complexing of TNP-Poly 18 to various immunogenic carriers failed to permit the development of an anti-Poly 18 antibody response in nonresponder strains. A defect in B cell function in nonresponders to TNP-Poly 18 was excluded by showing that anti-TNP antibody responses could be elicited by TNP-protein conjugates in nonresponders, even after pre-exposure to TNP-Poly 18. These results thus suggest that *Ir*-TNP-Poly 18 genes are not expressed at the B cell level, and that their action at the T cell level does not involve suppression. Brown *et al* (1979) showed apparent *Ir* gene expression by APC from responder and nonresponder strains, in the *in vitro* T cell proliferative response. This study does not, however, address the possibility of concurrent *Ir* gene control of T cell function.

Rather different results were obtained in the guinea pig. Strain 13 animals failed to respond with DTH or anti-Poly 18 antibody, but did give an anti-TNP response on challenge with high doses of TNP-Poly 18 *in vivo*. Strain 2 animals, by contrast, gave DTH to Poly 18 and anti-TNP antibody, but failed to respond with antibody to the Poly 18 backbone. This suggests the possibility that *Ir* genes may be expressed at the B cell in strain 2 animals, and at other site(s) in strain 13. Unlike the situation in the mouse, conjugation of TNP-Poly 18 to an immunogenic carrier (BOC-Gly-ARA-Tyr) could elicit DTH and antibody to both antigen and carrier determinants in strain 13 animals. This result is compatible with an APC defect in this strain, and, further, an antigen-specific T cell defect cannot be excluded.

Studies with antigens of this series were continued by Singh *et al* (1980). A dissociation between the ability to prime for antibody and T cell proliferation on the one hand, and DTH on the other, was observed in the mouse; the nonapeptide [Glu-Tyr-Lys-(Glu-Tyr-Ala)$_2$] being sufficient for induction of the former, while the latter requires the corresponding dodecapeptide. Elicitation of these responses required challenge with the prototypical octadecapeptide. Thus, a response by effector cells has somewhat different antigenic requirements than priming. Differences in the size and nature of the antigenic determinants recognized by the cooperating cell types, as suggested by Benacerraf (1978), may partially account for this observation. By this view, B cells recognize conformational, as opposed to sequential, determinants, the latter constituting the T cell repertoire. Peptide antigens do not assume alpha-helical configuration at much below the nonapeptide to dodecapeptide; hence B cell activating determinants would not be expressed on smaller fragments. Thus, T cell *Ir* genes may act at the level of recognition of the primary amino acid sequence, rather than on topographical determinants. Although this interpretation is historically favoured (Mozes *et al*, 1974; Keck, 1975b; Rosenthal, 1978; Sachs *et al*, 1978), the possibility that T cells may recognize topographical determinants has been strongly raised by work done recently with naturally-occurring proteins (Solinger *et al*, 1979; Okuda *et al*, 1979) (*vide infra*).

Ir gene control of responses to heterologous proteins

Certain proteins are highly conserved in phylogeny, and thus show at most slight differences in primary amino acid sequence, even between quite unrelated species. Examples of these would include the insulins, various respiratory pigments such as the cytochromes, and oxygen transport proteins (myoglobin). In a number of instances, these variant proteins have been found to be immunogenic, with the responsiveness under H-2 linked *Ir* gene control. Given the close structural relationships to homologous proteins, it follows that the number of antigenic determinants recognized as foreign must be extremely limited under these conditions. Hence, a number of groups have exploited this property in studies of the nature of the determinants recognized by T cells, and antibody molecules.

The work of Arquilla and Finn (1963, 1965) and of Arquilla *et al* (1967), showing that anti-insulin antibodies from different guinea pig strains react with different parts of the insulin molecule, has already been mentioned. Barcinski and Rosenthal (1967) extended these early studies, using the *in vitro* T cell proliferation assay to map the insulin determinants recognized by guinea pig T cells. Strain 13 cells were shown to respond to a portion of the insulin B chain, and the response was determined by the guinea pig MHC. Strain 2 T cells, by contrast, recognize the A chain loop, residues A8, A9, and A10 being crucial.

Keck (1975a, b) mapped the T cell activating determinants of beef insulin by *in vivo* immunization with DNP-insulin. Under these conditions, the A chain loop is the principal determinant recognized in mice of the H-2*a* haplotype. Pork insulin, which is homologous with mouse insulin in the A chain loop, is not immunogenic in this mouse strain. Mice of H-2*d* haplotype obviously recognize a different determinant, as both beef and pork insulin are immunogenic in such strains, while animals of *k* and *s* haplotypes are strict nonresponders to either.

Rosenwasser *et al* (1979) extended these results to H-2*b* mice, and further localized the determinant recognized by H-2*d* animals to the N-terminal eight amino acids of the B chain. The *Ir* gene for insulin in mice was mapped to I-A, by the use of recombinant strains.

Rosenthal *et al* (1977) obtained evidence suggesting that a major role of the APC was determinant selection. Insulin-immune strain (2 x 13)F_1 T cells were shown to comprise a strain 2-restricted population recognizing the A chain loop, and another, restricted to the B chain determinant in association with strain 13 APC.

Schwartz *et al* (1978) and Solinger *et al* (1979) carried out similar analyses of the immune response to pigeon cytochrome *c*, identifying the principal determinant responsible for immunogenicity in the mouse, and showing that its recognition is controlled by dual complementing *Ir* genes. The amino acid residues contributing to the determinant were identified by comparison of the ability of various heterologous cytochromes to stimulate pigeon cytochrome *c* immune T cells *in vitro*, and by the use of CNBr cleavage fragments of cross-reacting cytochromes; three amino acids, spaced well apart in the primary sequence, were found. In the folded molecule, however, these

three residues are in close proximity, and can form a topographical determinant having a span of some 12 Angstroms.

Okuda *et al* (1978) have extensively studied the immunological properties of sperm whale myoglobin, showing that the T cell proliferative response to this antigen is under the control of at least two H-2-linked *Ir* genes. A total of five antigenic sites in the intact molecule were identified by their ability to elicit goat or rabbit antibodies, and each was found to be a topographical determinant. Synthetic peptides of the correct primary sequences were then synthesized. It was shown that the *in vitro* proliferative response of primed cells to each of these determinants was under the control of H-2 linked *Ir* genes, and that some genes mapped to I-A alone, and others to I-A and I-C. These results are consistent with the notion that the overall immune response to a complex antigen is the sum of the responses to individual, *Ir* gene controlled determinants.

Finally, Sachs *et al* (1978) have used the bacterial enzyme, Staphylococcal nuclease, as a model *Ir* gene controlled antigen. This differs from the genetic variant heterologous proteins described above, in that responsiveness maps to the I-B subregion of H-2, and also to non-H-2 background genes. The non-H-2 genes regulate the ability to hyperimmunize an animal, and can override the effects of the H-2 linked genes. The immunogenicity of fragments of the enzyme was determined, and, in contrast to the complex genetic control of the response to the native protein, one particular fragment elicited an all-or-none response. The all-or-none pattern of reactivity to this determinant persisted even in hyperimmune sera, in which total anti-nuclease activities were comparable. Recombinant mouse strains on a B10 background were used to map the *Ir* genes controlling the T cell proliferative response to nuclease (*Ir*-Nase) to the I-B subregion, and evidence for other H-2-linked *Ir* genes was found. The response to individual determinants on the nuclease molecule was shown to be under multiple H-2-linked *Ir* gene control, in experiments in which peritoneal exudate T cells primed to the whole nuclease molecule were stimulated *in vitro* by various fragments. Interestingly, T cell proliferation was optimally stimulated by fragment concentrations equivalent (in molar terms) to those for intact nuclease, while the affinity of antibody for fragments was 1000 to 10000 times less than for the native protein. This suggests, albeit indirectly,

that T cells recognize sequential determinants, while B cells have specificity for antigen conformation.

F. Major histocompatibility complex control of cell interactions

Closely related to this last section is the entire question of H-2 control of cell interactions in the immune response. I have already described the early experiments leading to the definition of the cooperating cell types in the immune response (Section D., above), and the evidence for recognition of hapten and carrier moieties by distinct cells. In many of these studies, it was found that *in vivo* cooperation between thymus and bone marrow derived cells was possible only if they were syngeneic or H-2 compatible (Miller and Mitchell, 1969; Claman and Chaperon, 1969).

Kindred and Shreffler (1972) confirmed these results, showing that immunological competence could be induced in nude mice backcrossed repeatedly to Balb/c, by syngeneic or H-2 compatible, but not not by allogeneic thymocytes. As the nudes are incapable of rejecting the transferred cells, it follows that the H-2 complex plays an important role in permitting cell cooperation.

Katz *et al* (1973a, b) extended these earlier results in an extensive series of experiments, showing that antigen-specific helper T cells could cooperate specifically with syngeneic, but not allogeneic B cells in giving a secondary IgG response to hapten-carrier conjugates. Further, suppression mediated by negative allogeneic effects was excluded, as the presence of histoincompatible T cells did not interfere with cooperation between syngeneic T and B cells (Katz *et al*, 1974, 1976b). The genes controlling cooperation were mapped to within H-2 (Katz *et al*, 1973b), and further to the I-A subregion (Katz *et al*, 1975).

At about the same time that Katz described an H-2 influence on T-B cell cooperation, Rosenthal and Shevach (1973) demonstrated a similar requirement for APC - T cell genetic identity in the *in vitro* T cell proliferative response in the guinea pig. Katz *et al* interpreted these results as implying the existence of cell surface receptors distinct from those for antigen, and which are crucial in permitting effective (T-B) cell interaction. Genetic identity between the cooperating cells was necessary to optimize the interaction between these *cell interaction* (CI) molecules (Katz and Benacerraf, 1975),

and their respective acceptors.

Evidence for an analogous MHC restriction of the interaction between cytotoxic T lymphocyte (CTL) and target cell was obtained for virus-infected (Zinkernagel and Doherty, 1974), chemically-modified (Shearer, 1974), and minor histocompatibility (H) antigen bearing targets (Bevan, 1975; Gordon *et al*, 1975), and mapped to the K and D regions of the MHC (Blanden *et al*, 1975).

From these quite different model systems arose two distinct hypotheses on the mechanism of MHC-restricted cell interactions. The Katz results were interpreted in terms of a dual recognition hypothesis, with physically distinct antigen receptors and Cl molecules and acceptor sites on the cell surface. By contrast, the CTL studies (particularly those of Shearer, 1974), led more easily to an 'altered-self' model of T cell recognition, in which T cells possess a single receptor for an entity comprising a self-marker, modified by association with extrinsic antigen (Zinkernagel and Doherty, 1974). A great deal of effort has been expended in attempting to resolve between these two alternatives, without much perceptible result. I find it unreasonable to expect that I-region restricted, and (K,D)-restricted interactions should necessarily have a common mechanism, and think it plausible that both may co-exist. A two-receptor model has a considerable advantage in explaining many of the results seen in T cell - APC restriction, and in addition lends itself better to explaining certain findings in chimaeric systems (*vide infra*), as well as providing a general model for genetic control of cell interactions.

The validity of the original model of Cl molecule control of cell interactions came into doubt soon after it was proposed, on the basis of studies showing that T-B cell interactions are not genetically restricted under all circumstances.

The first evidence for this possibility came in the work of Bechtol *et al* (1974), with tetraparental mice derived by fusion of embryos of HR and LR strains to (T,G)-A--L. These animals were claimed to produce anti-(T,G)-A--L antibody of both high and low responder allotypes on *in vivo* immunization, a finding which was interpreted in terms of T - B cell cooperation across H-2 differences, particularly as *Ir-1* genes were thought to be expressed only in T cells. These findings could not, however, be verified in subsequent work by the same (Press and McDevitt, 1977) or other (Warner *et al*, 1978) groups. Only high responder B cells were found to be activated under these conditions.

Better evidence came in studies with bone marrow chimaeras $A + B \rightarrow (A \times B)F_1$, reported by von Boehmer *et al* (1975a). T lymphocytes of a single parental haplotype from chimaeras were found to cooperate efficiently *in vivo* with parental B lymphocytes of either haplotype. As the cells from the chimaeras were shown to be mutually tolerant (von Boehmer *et al*, 1975b; Sprent *et al*, 1975), it was proposed that mutual tolerance in the cooperating cell populations was sufficient to allow cooperation across H-2 differences, and that 'H-2 restriction' might simply reflect the effects of unappreciated alloreactivity between H-2 different cells (von Boehmer *et al*, 1975a).

Adaptive differentiation

In light of these results, Katz proposed the theory of *adaptive differentiation*, to explain the triple paradox of strict T - B cell restriction for normal cells; the absence of such restriction for tolerant cells, and the lack of alloreactivity sufficient to account for this difference (Katz *et al*, 1976a, 1977b). In outline, this theory maintains that during ontogeny, lymphoid precursors 'learn' the CI molecule compatibilities necessary for productive cell interactions, and that this learning is directed by the MHC phenotype of the environment. Initial results (Katz *et al*, 1976a) suggested that this model was valid, at least for B lymphocytes, although the possibility of pre-existing, parentally restricted lymphocyte subpopulations in F_1 cells was not excluded.

The analysis of adaptive differentiation and like events has recently become one of the major preoccupations of cellular immunology. Against an adaptive differentiation-like model, are the results of Waldmann *et al* (1977), and of Swain *et al* (1977), describing inhibitory T cells activated by MHC differences, and capable of preventing cooperation between allogeneic T and B cells *in vitro*. This is in direct contradiction to the *in vivo* cell mixing experiments of Katz *et al* (1974, 1976b). Elimination of the inhibitory cells by irradiation allowed successful interaction between semiallogeneic, but not fully allogeneic T and B cells. Thus these authors claimed H-2 restriction to be largely artefactual, and a reflection of inadequate attention to eliminating possible sources of alloreactivity.

Waldmann *et al* (1975) obtained similar results to von Boehmer *et al* (1975a), using, however, an *in vitro* assay system to examine the H-2 restriction patterns of cells from tetraparental bone marrow chimaeras, and showing (again) unrestricted cooperation

across H-2 barriers. Cells from one way bone marrow chimaeras *A* -> (*A* x *B*)F_1, by contrast, were restricted to self-H-2 *in vitro*. The difference between one-way and two way bone marrow chimaeras was attributed at the time either to incomplete tolerance, or the absence of a mechanism to preserve T cell clones able to cooperate across H-2 barriers in one way chimaeras.

Subsequently, Waldmann *et al* (1976) carried out experiments to examine the influence of the T cell priming environment on cooperative preferences. Unprimed cells of strain *A* from *A* + *B* -> (*A* x *B*)F_1 tetraparental bone marrow chimaera were primed for seven days in an *A* environment. If the priming environment is the sole determinant of the cooperative preferences of these cells, restriction to cooperation with strain *A* B cells would be expected. In the event, these cells were found to cooperate with both *A* and *B* effectors, precisely as with *in situ* priming. This result is difficult to reconcile with an 'altered-self' model; however, it fits well with a dual recognition scheme. Additionally, it argues against the priming environment having much influence on the expression of patterns of cooperation, instead assigning this role to the environment in which differentiation occurs.

Adoptive priming of chimaeric cells over a longer period of time in irradiated animals of *A* genotype resulted in the loss of unrestricted cooperation, and the development of a strict preference for the priming host genotype. The latter results are quite amenable to explanation in terms of the adaptive differentiation hypothesis of Katz and Benacerraf (1975).

Influence of the thymus

The entire field of H-2 restriction underwent a re-evaluation with the work of Zinkernagel and his collaborators on the generation of CTL against virus-infected targets by cells derived from bone marrow chimaeras. Zinkernagel (1976) showed a functional dichotomy in T cells from one-way BMC *A* -> (*A* x *B*)F_1, clonal subsets of these *A* cells being able to lyse either *A* or *B* virus infected targets. Pfizenmaier *et al* (1976) and von Boehmer and Haas (1976) obtained equivalent results with virus infected and chemically-modified target cells. These results are all compatible with the interpretation that the T cell recognition repertoire is acquired, and depends on the environment in which T cells are sensitized under H-2 tolerant conditions. These early studies did not

directly address, however, the nature of the antigenic determinant recognized by the T cell, and thus could not distinguish between one and two receptor models. Evidence for a two receptor model in the (K,D)-restricted viral system came in the studies of Zinkernagel. A requirement of any two receptor model is that specificity for the restricting determinants be acquired independent of antigen exposure (von Boehmer *et al*, 1978; Langman, 1978); while selection in a one-receptor model should be antigen dependent (Doherty and Zinkernagel, 1974). Radiation and thymus-grafted chimaeras were used to address this point, and to identify the elements acting to impose H-2 restriction.

Low level chimaerism was induced in neonatal animals by injection of semiallogeneic lymphoid and stem cells, as described by Billingham *et al* (1953). After sensitization to virus either *in situ* (Zinkernagel *et al*, 1977) or in a lethally-irradiated F_1 host (Zinkernagel *et al*, 1978a) CTL could be induced only to syngeneic virus-infected targets, but not to allogeneic targets. Thus, tolerance to H-2 alone is not sufficient to allow generation of CTL with specificity for the tolerated alloantigen. Comparable results were obtained in the helper T cell model for antibody by Kindred (1975), who attempted (unsuccessfully) to reconstitute nude mice with neonatally tolerant T cells, and by Sprent and von Boehmer (1976), who reported failure of cooperation between B cells and allogeneic T cells depleted of alloreactivity by *in vivo* negative selection.

Irradiation chimaeras ($A \times B)F_1$ -> A or B; or A or B -> ($A \times B)F_1$ were similarly used to determine the influence of the chimaeric host on restriction specificity. F_1 -> A chimaera CTL behaved exactly like the corresponding A CTL, in that they were restricted to lysis of A but not B targets (Zinkernagel *et al*, 1978b, c). Bevan (1977) independently arrived at a similar conclusion in the minor H CTL system.

Irradiated parental host reconstituted with adult F_1 spleen, as opposed to stem cells, gave rise to CTL capable of lysing virus infected targets of both parental haplotypes, indicating that the host influence is imposed on CTL specificity only during maturation, and not on fully mature T cells.

A role for the thymus in this selection was suggested by previous work showing its influence on T cell maturation (see Stutman *et al*, 1977). Thus, thymus-grafting experiments were carried out to determine whether MHC restriction patterns were

dictated by the thymic haplotype. (*A* x *B*)F_1 animals were thymectomized as adults, irradiated, and reconstituted with syngeneic bone marrow. After reconstitution, they were grafted with irradiated *A* or *B* thymus tissue, and allowed to recover for several months. These animals gave CTL responses only to virus infected targets syngeneic with the thymus graft used for reconstitution (Zinkernagel, 1978). Thus, the radioresistant (?epithelial; Weissman *et al*, 1978) tissues of the thymus determine the restriction specificities of the cells differentiating through them.

These studies did not allow conclusions on whether thymic influence was positive (ie. selection) (Bevan and Fink, 1978; von Boehmer *et al*, 1978; Langman, 1978; Zinkernagel, 1978) or negative (suppression) (Blanden and Ada, 1978). To address this, (*A* x *B*)F_1 nude mice were grafted with *A* and/or *B* thymuses, and the ability to generate CTL to *A* or *B* targets was determined. Suppression would be predicted to result either in functional immunoincompetence, or restriction to one haplotype, depending on which suppressor was dominant, in the presence of two histologically intact thymus grafts; while a positive selection role for the thymus should result in the expression of both specificities. In the event, evidence for positive selection was obtained, although the results are, frankly, unconvincing (Zinkernagel *et al*, 1979). Smith and Miller (1980), however, concluded that suppression could be shown.

Finally, the involvement of peripheral reticuloendothelial and lymphohaemopoietic cells in peripheral T cell maturation was suggested by the observation that *A* -> (*A* x *B*)F_1 or (*A* x *C*) -> (*A* x *B*)F_1 T cells were ordinarily restricted to lysis of *A* targets, but could be induced to lyse *A* and *B* targets by adoptive priming in (*A* x *B*)F_1 virus infected hosts. To account for this observation, it was necessary to postulate (Zinkernagel *et al*, 1978a) that the dose of irradiation used to prepare the (*A* x *B*)F_1 recipients of stem cells was not sufficient to immediately eliminate all F_1 RE cells, but that sufficient of them persisted for long enough to allow differentiation of both *A* and *B* restricted CTL precursors. By the time of priming, these residual F_1 cells would have been replaced by donor-derived RE cells, and consequently only the donor-restricted subpopulation would be expanded under normal circumstances. Adoptive priming in the presence of (*A* x *B*)F_1 APC, however, allows expression of both restriction specificities. Some evidence for this view was obtained by the use of very high doses of irradiation in the preparation of the

chimaeras; see Zinkernagel *et al* (1978a), and Zinkernagel (1978).

As with any system in which such apparently clear-cut results are obtained, though, it was not long before exceptions to these principles were described.

Thus Bevan (1977) showed a *preference* for recognition of modified host target cells in (*A* x *B*) -> *A* radiation chimaeras, rather than the absolute restriction reported by Zinkernagel. Similarly, Matzinger and Mirkwood (1978) reported that fully H-2 incompatible (but congenic) radiation chimaeras not only were immunocompetent (cf. Zinkernagel, 1978), but also that they showed CTL responses to minor H antigens associated with both donor and host H-2 types, although with a clear host bias. Various possible explanations for this result were considered, but the most likely is simply that vigourous priming is sufficient to expand up pre-existing donor restricted CTL precursors to significant levels.

Doherty and Bennink (1979) used *in vivo* negative selection to H-2 to deplete alloreactive T cells to a given haplotype. These filtered T cells were found to be able to recognize virus-infected cells of some unrelated haplotypes but not of others, suggesting that 'aberrant recognition' may be significant in this. According to this view, T cells specific for (say) haplotype *A* + virus *X* may in fact recognize the same determinant as expressed by *B* + virus *Z*. This is compatible only with altered-self recognition, and requires an unacceptably large redundancy in T cell receptor specificities.

Blanden and Andrew (1979) described the existence of CTL specific for virus-infected targets of *B* haplotype in a large proportion of (*A* x *B*) -> *A* chimaeras, although some animals showing absolute restriction to host H-2 were also noted. Their interpretation of these results was based on a negative selection model for thymic function, which is improbable *a priori*.

These results thus suggest that the thymus and reticuloendothelial systems are involved in a very fundamental way in determining the H-2 restriction patterns of cytotoxic T lymphocytes. Many groups have since attempted to extend these findings to other experimental systems showing analogous genetic control. Chief amongst these is the I region determined interaction between T cells, B cells, and APC in T cell proliferation or antibody formation *in vivo* or *in vitro*.

In view of the complicated nature of these findings, I propose to discuss the results obtained by the various groups according to cell interactions.

T cell - macrophage interactions in helper T cell induction

The first evidence for MHC control of the interaction between sensitized T cells and antigen presenting cells was obtained by Rosenthal and Shevach (1973). These authors showed that T lymphocytes from Strain 2 or Strain 13 guinea pigs primed to PPD could be stimulated to proliferate only by syngeneic antigen-pulsed APC. Suppression or suppressive allogeneic effects were excluded as the basis of the failure of allogeneic APC to activate cell proliferation. Additionally, alloantisera directed against cell surface MHC antigens effectively blocked the T cell - APC interaction, arguing that these molecules are important in the generation of a proliferative response. The requirement for physical proximity between T cell and APC was clearly shown by Lipsky and Rosenthal (1973, 1975) who delineated two types of APC - lymphocyte interaction; one reversible and antigen-independent, and the other, a stable antigen-dependent association leading to clonal proliferation of the lymphocyte.

Subsequent studies by Paul *et al* (1977) indicated the existence of distinct clonal MHC restricted subpopulations in normal F_1 guinea pig T cells, each able to cooperate with only one of the parental APC populations. Positive and negative *in vitro* selection was used to enrich for the restricted subpopulations, and it was established that the selective responsiveness did not reflect the action of alloantigen-specific suppressor cells, by double-negative selection to antigens and MHC products.

The results obtained in these experiments are compatible with the concept of mutual self-recognition of cell interaction molecules by the cooperating cell types. Nonetheless, the existence of distinct parentally-restricted subpopulations of F_1 T cells requires either allelic exclusion of CI molecules on the cell surface, or preferential association of the allelic forms of CI molecules with antigen receptors having particular specificities.

Equally, these results fit with either one receptor ('altered-self') or two receptor models of T cell recognition. The latter hypothesis is difficult to reconcile, however, with the findings of Greineder *et al* (1976) and Ellner *et al* (1977), that T cell proliferation can be blocked only by anti-Ia antiserum, and not by antibodies against antigen. Additionally,

dual recognition does not adequately account for *Ir* gene expression by the APC. To get around this difficulty, Rosenthal *et al* (1975, 1977) proposed that T lymphocytes are not themselves specific for Ia molecules (*Ir* gene products), but that the *Ir* genes function at the APC level, by a process of 'specificnt determinant selection.'

Erb and Feldmann (1975a, b, c, d) and Erb *et al* (1976) were the first to extend the observation of T cell - APC restriction to the mouse. Initially, they identified an APC requirement for helper T cell induction *in vitro*, and showed genetic restriction for effective T cell - APC interactions in the presence of soluble antigen. For soluble antigens, APC could be replaced by an antigen-specific soluble factor derived from syngeneic but not allogeneic APC cultured with antigen; while for particulate or immobilized antigens, genetic restriction at that level did not apply. Based on these results, they proposed at least two pathways for helper T cell induction, depending on the physical state of the antigen. Additionally, direct contact between T cells and APC was not necessary for helper cell induction, arguing that soluble factors may play a physiological role (Erb and Feldmann, 1975a, b). Genetic control of the interaction for soluble antigens was localized to the I-A subregion of H-2, and suppressive effects were excluded (Erb and Feldmann, 1975c). Finally, the nature of the soluble macrophage replacing factors was investigated. A genetically related macrophage factor (GRF) was identified as being produced by purified APC incubated with soluble antigen, and was able to induce helper cells from syngeneic precursors in the absence of further APC or antigen. A nonspecific macrophage factor (NMF), capable of replacing APC function in helper cell induction only for particulate antigens, could be obtained from syngeneic or allogeneic macrophages cultured without antigen. GRF was shown to contain Ia antigens linked noncovalently to a small antigenic determinant, and to have an overall molecular weight of approximately 50000 daltons (Erb *et al*, 1976); the nature of its interaction with the T cell in helper induction is unknown, and its existence is compatible with either one or two receptor models for T cell recognition.

Erb *et al* (1978) analysed this point further, using *in vitro* induction of helper T cells by antigen and APC. F_1 T cells were found to give rise to helper T cells capable of interacting with B cells of either parental haplotype, provided the same APC were used for the induction of help and in the cooperation cultures. Similar findings were reported

by Thomas and Shevach (1977), with F_1 guinea pig T cells in a macrophage-dependent *in vitro* proliferation assay, and by McDougal and Cort (1978). These results make an unmodified CI molecule hypothesis rather less tenable, as they require allelic exclusion of CI molecules on different syngeneic T cell subpopulations.

Evidence for H-2 restriction prior to priming was obtained by Erb *et al* (1978), with T cells from *A* + *B* -> (*A* x *B*)F_1 bone marrow chimaeras. Unprimed T cells of one haplotype (say, *A*) prepared by alloantiserum treatment, were unable to give rise to helper cells restricted to *B* haplotype, on *in vitro* incubation with APC of *B* haplotype. As the cooperation cultures contained appropriate APC and B cells, this implies that the T cell - APC interaction, in the absence of alloreactivity, is restricted in primary, as well as secondary, responses *in vitro* (*vide infra*). This result is not compatible with those obtained by *in vivo* priming (Erb *et al*, 1979), in which unrestricted cooperation was shown.

In subsequent studies, T cells from *A* -> (*A* x *B*)F_1 chimaeras were found to be restricted to cooperation with syngeneic APC in the *in vitro* induction of helper T cells. Similarly, unprimed F_1 -> parent chimaeric T cells were restricted to parental APC for helper cell induction (Erb *et al*, 1979). Based on these results, the authors postulated the existence of two stages in the development of competence in helper cells, one thymus-dependent, and the other requiring interaction with macrophage-like cells. In parent -> F_1 chimaeras, thymic influence is permissive for APC-mediated restriction, while in F_1 -> parent chimaeras, thymic elements are restricting.

Singer and his colleagues have obtained strong evidence for H-2 restriction of the T cell - APC interaction, using *in vitro* and *in vivo* induction and assay of helper cell function. It was found that unprimed normal (*A* x *B*)F_1 and *A* -> (*A* x *B*)F_1 chimaeric T cells showed free cooperation with either *A* or *B* APC; while (*A* x *B*)F_1 -> *A* T cells were restricted to *A* APC, or to H-2 recombinant APC expressing *A*-type I-A determinants (Singer *et al*, 1979). The restricted cooperation between F_1 -> parent chimaeric T cells and APC was shown not to reflect suppression by allogeneic APC (Hodes *et al*, 1980), and thus is consistent with either positive or negative selection models of thymic function. Subsequent studies in which limiting numbers of normal (*A* x *B*)F_1 T cells were used to generate primary *in vitro* helper T cells verified the finding of T cell - APC but

not T - B cell genetic restriction (Singer *et al*, 1980). These results are especially important as they overcome certain objections with respect to the use of highly manipulated parent -> F_1 chimaeric cell populations.

H-2 restriction of the induction of T helper cells *in vivo*, by contrast, has been less thoroughly examined. Gorczynski *et al* (1971) obtained evidence that adherent cells were necessary for the induction of *in vivo* immune responses to SRBC, and that these adherent cells had physical properties identical to those of the A cells active in *in vitro* assays (Shortman *et al*, 1970). The immunological specificity of these cells was not examined, however, nor was the genetics of reconstitution of immunological competence.

Much of our understanding of the latter point has come in the work of Sprent and his colleagues, and has relied heavily on *in vivo* positive or negative selection of T cells to antigen, in the context of APC of different genotypes. Thus, when normal animals are injected intravenously with large amounts of antigen, reactivity of recirculating thoracic duct lymphocytes to the antigen is abrogated within 24 hours (Rowley *et al*, 1972; Sprent *et al*, 1972, 1974). This abrogation reflects a transient sequestration of reactive cells in the spleen (Sprent and Lefkovits, 1976), and is followed by the appearance of large numbers of specifically-reactive cells in the thoracic duct lymph. Similar findings have been reported in adoptive transfer systems, in which lymphoid cells and antigen are given intravenously to irradiated hosts (Ford and Atkins, 1971; Sprent, 1978).

Based on the *in vitro* findings, it may be supposed that the T cell sequestration seen *in vivo* represents a need for stimulation by particular APC, and therefore, that this process might be H-2 restricted. Sprent (1978a) verified this prediction for both positively and negatively selected homozygous T cells filtered through syngeneic recipients along with SRBC. Thus, SRBC specific T helper cells were substantially depleted in TDL collected one day after transfer into irradiated hosts, while by 5 days, substantial enrichment for SRBC help was seen. Selection did not affect the levels of help for unrelated antigens.

This result thus suggests that particulate antigens must be degraded by the host, and presented in association with host determinants, for effective activation of T cells. Undegraded antigen is most likely ignored by the system. T cells recognizing the

association between host cell and antigen are activated, and sequestered transiently in the spleen, where they undergo extensive proliferation before appearing in the periphery.

The results obtained with *in vitro* selection of heterozygous F_1 T cells in both guinea pig and mouse (Paul *et al*, 1977; Swierkosz *et al*, 1978) were also verified by Sprent (1978a, b, c, d) in the *in vivo* system. Thus, (*A* x *B*)F_1 T cells negatively selected to antigen in strain *A* animals failed to cooperate with strain *A* B cells *in vivo*. Conversely, the same cells taken at 5 days (positive selection) showed preferential cooperation with strain *A* B cells, and gave responses characteristic of unprimed cells to strain *B* B cells. The simplest interpretation of these findings is that only the *A* restricted T cell subpopulation undergoes selection to antigen, and hence comes to outnumber the *B* restricted subpopulation.

Evidence against this apparent requirement for T cell recognition of identical MHC determinants on B cells and APC was obtained by Singer *et al*, (1979), with *in situ* primed (*A* x *B*)F_1 T cells, and parental B cells. Under these conditions, secondary *in vitro* responses were possible between allogeneic B cells and APC.

These results also clearly imply that the cell responsible for selection is derived from the irradiated host, rather than the T cell donor. Indeed, supplementation of an *A* host with T cell depleted *B* spleen cells results in selection for both *A* and *B* restricted subpopulations of F_1 T cells. Further studies have shown that this restricting cell is radioresistant, nylon wool adherent, Thy 1 negative, present in spleen and peritoneal exudate cells, rare in lymph nodes, and absent from TDL (Sprent *et al*, 1980). Thus it fulfills most of the criteria for a cell of the macrophage lineage, and certainly is equivalent to the A cell of Gorczynski *et al* (1971). Kappler and Marrack (1976) obtained equivalent results, priming F_1 mice *in situ* with KLH pulsed parental PEC, and examining the cooperative potentials of these cells *in vitro*, with parental or F_1 B cells. Restriction of cooperation to B cells syngeneic with the priming APC was observed, suggesting that selection to APC haplotype and antigen was involved.

Thus, the results fit with the general prediction that normal homozygous T cells, even when fully depleted of alloreactivity, are restricted to cooperation with APC bearing antigen in association with syngeneic MHC determinants. Heterozygous T cells, by contrast, are capable of interaction with cells of either of the respective parental

haplotypes, after appropriate *in vivo* or *in vitro* selection.

As suggested above for the viral system, quite different considerations apply to homozygous T cells which have undergone differentiation in a heterozygous environment. Thus, Waldmann *et al* (1975) and von Boehmer *et al* (1975a) showed cooperation across H-2 differences for homozygous T cells derived from tetraparental bone marrow chimaeras $A + B \rightarrow (A \times B)F_1$, with *in vitro* and *in vivo* assay systems, respectively. This together with the work of Swain *et al* (1977) suggests that tolerance of H-2 antigens is insufficient to allow unrestricted cooperation. However, T cells from one-way bone marrow chimaeras (Waldmann, 1977), and neonatally-tolerant donors (Waldmann *et al*, 1978) were restricted to cooperation with self-H-2, despite apparently complete tolerance. Marusic *et al* (1977) obtained similar results with a tumour rejection system. Thus, unless operating at a very subtle level, suppression is not adequate to account for failure of alloegeic cooperation. In light of the work of Kappler and Marrack (1976), and of Sprent (1978a), suggesting the influence of the priming environment on restriction patterns of normal F_1 cells, Waldmann *et al* (1976, 1978) examined the ability of one-way chimaeric and neonatally tolerant cells to be primed in syngeneic and allogeneic environments. It was hypothesized (Waldmann, 1978), that the restriction seen in $A \rightarrow (A \times B)F_1$ chimaeras primed *in situ* was simply a reflection of the presence of APC of only one haplotype, and that this deficit could be overcome in an appropriate priming environment. In early experiments (Waldmann, 1976) results apparently excluding (in their interpretation) any influence of the priming environment were obtained; T cells from one-way or two-way bone marrow chimaeras could be primed in a homozygous environment to cooperate in an unrestricted fashion with B cells. Subsequently, the techniques for adoptive priming were optimized so as to maximize the influence of the chimaeric host, and preference for the haplotype of the priming environment could be shown for both one-way and two-way BMC. Neonatally-tolerant T cells could not, however, be primed in an allogeneic environment. These results together suggest the functional equivalence of T cells from one and two way chimaeras (apart from *in situ* priming environment), and also imply that the tolerant state in chimaeras is quite different from that in neonates.

Identical results to the above were obtained by Sprent and von Boehmer (1979), with parental T cells from *A* + (*A* x *B*)F_1 -> (*A* x *B*)F_1 bone marrow chimaeras, and by Sprent with T cells from one way bone marrow chimaeras (Sprent *et al*, 1980), both studies using the technique of *in vivo* positive selection to antigen. F_1 -> parent chimaeras were subsequently used to examine restriction patterns imposed on T cells independent of priming, which necessarily occurs with F_1 APC. Much as was seen by Zinkernagel *et al* (1978a, c) for CTL, helper T cells showed preference for B cells identical to the parental host (Waldmann *et al*, 1978; Sprent, 1978e). Additionally, thymus grafting experiments were carried out to verify that H-2 preference is determined by the thymus. (*A* x *B*)F_1 ATxBM animals were reconstituted with *A* or *B* thymus grafts, and the cooperative preferences of the (*A* x *B*)F_1 cells determined. Restriction to cooperation with B cells syngeneic to the thymus graft was found (Waldmann *et al*, 1979), consistent with the Zinkernagel results for CTL (*vide supra*).

These experiments thus carry with them the strong suggestion that analogous differentiation processes occur in the thymus for both CTL and helper cells for antibody, despite the quite obvious differences between the experimental systems.

In particular, the data are consistent with a predominant thymic influence on cell cooperative preferences, although the expression of these preferences is modified in the periphery by contact with antigen associated with cells of the reticuloendothelial system. Nonetheless, restriction antedates contact of the immune system with antigen, as seen for parent -> F_1 chimaeras primed adoptively; these animals have both 'anti'-*A* and 'anti'-*B* cell subpopulations, the 'anti'-*B* cells being generated at the expense of the normal alloreactive cells to *B*. Conversely, in F_1 -> parent chimaeras, the normal 'anti'-*B* subpopulation fails to develop, as the *B* haplotype is absent from the host thymus.

Katz (1980) has recently presented a scheme for adaptive differentiation in normal and chimaeric animals, which is capable of explaining most if not all of the above observations. In outline, it postulates that cells with high affinity for CI molecules are selected against in the host environment (normal or chimaeric), leaving behind low-to-moderate affinity cells capable of fruitful cooperative interactions. High affinity anti-CI cells for specificities not selected against effectively regulate the emergence of cells bearing those particular CI molecules, since such cells fulfill no particular purpose in

the system. These data also place at least one site of expression of H-2 restriction at the interaction between the T cells and reticuloendothelial cells; APC in the case of helper T cells. One must therefore consider the extent to which the H-2 restriction described at other cell interactions simply reflects distal effects of this primary restriction. Certainly most any of the results suggesting preferential cooperation of helper T cells with B cells syngeneic to the priming APC can be interpreted in this way.

T - B cell cooperation

The early evidence for H-2 restriction of T-B cell cooperation has been briefly summarized above.

Normal homozygous T cells

Katz and his collaborators have carried out extensive and systematic studies with an *in vivo* adoptive transfer system, concerning the ability of histoincompatible T and B cells to cooperate in giving an antibody response to hapten-carrrier conjugates (Katz *et al*, 1973a, b). These studies relied on the known radioresistance of T helper cell function *in situ* (Katz *et al*, 1970a). The initial approach taken was to transfer hapten-primed B cells into irradiated, allogeneic carrier-primed adoptive hosts (Hamaoka *et al*, 1973). It was shown, however, that sufficient alloreactive T cells remained in the host after irradiation to exert a strong positive allogeneic effect on transferred B cells. This allogeneic effect was manifested by apparently unrestricted T - B cell cooperation. To overcome this problem, a double adoptive transfer protocol was devised, in which an (*A* x *B*)F_1 animal was used as the adoptive host. Limiting numbers of carrier-primed T cells were transferred into a normal semiallogeneic host, which was sublethally irradiated 24 hours later, and given hapten-primed B cells intravenously, along with a hapten-carrier challenge. The delay of 24 hours between T cell transfer and irradiation was intended to allow migration of helper cells to recipient lymphoid organs, while the use of an F_1 host was based on the assumption that it is genetically incapable of reacting against either of the transferred lymphoid cell populations. The transfer of limiting numbers of cooperating cells should likewise minimize the chances of an allogeneic effect. Nonetheless, one must question the rather blithe transfer of whole immunocompetent spleen cell populations (as a T cell source) into an allogeneic host. Similar considerations applied to the design of *in vitro* experiments (Katz *et al*, 1973a).

Using this system, it was shown that H-2 incompatible T and B cells could cooperate in giving a secondary *in vivo* antibody response (Katz *et al*, 1973b). The same studies also indicated that non-H-2 background genes in the mouse strains did not determine the ability to cooperate. Subsequent studies, using appropriate recombinant strains, localized the genes controlling cell interactions to the I-region of the H-2 complex (Katz *et al*, 1975), and further, to the I-A subregion (Katz *et al*, 1976a).

Janeway *et al* (1976) used a particularly elegant system, involving the *Ir* gene for responsiveness to IgA myeloma protein (which maps to I-A), to arrive at a similar conclusion. T and B cells from mouse strains sharing only the I-A subregion were able to cooperate efficiently in giving a response to TNP-IgA, indicating the importance of this region in cell-cell interactions.

The experiments did not, however, rule out the possibility that failure of physiological cooperation of allogeneic cells might reflect the operation of subtle suppressive phenomena, particularly in light of Gershon's (1972) finding of ready generation of suppressor T cells in allogeneic lymphocyte mixtures. To test this, the ability of allogeneic carrier-primed T cells to interfere with cooperation of syngeneic carrier primed T cells, and hapten-primed B cells was determined in the adoptive transfer system. No evidence for suppression was obtained (Katz *et al*, 1974). Identical results were obtained in more extensive studies of the same sort (Katz *et al*, 1976b). Skidmore and Katz (1977) carried out analogous experiments, in which the ability of primed T cells to help mixtures of syngeneic and allogeneic B cells was assessed. Not only did the presence of allogeneic B cells not interfere with the response, but the PFC were all derived from the B cell population syngeneic with the T cells.

Finally, Katz (1977a) showed that pretreatment of parental host animals with antithymocyte serum abrogated their ability to mediate an allogeneic effect against allogeneic B cells, and also to provide 'help' to allogeneic, but not syngeneic, B cells. This result was interpreted as indicating that any apparent help provided to allogeneic B cells by a carrier-primed host most likely reflected nonspecific alloreactivity, rather than carrier-specific cooperation across H-2 differences.

Tolerant homozygous T cells

A number of other approaches have been taken to analyze the cooperative preferences of homozygous T cells, besides the use of irradiation *in situ*, to deplete alloreactive cells, without affecting helper function. Acute selection procedures, typically involving *in vivo* filtration through a heterozygous recipient (Ford and Atkins, 1971; Sprent and von Boehmer, 1976); neonatal tolerance induction (Kindred, 1975; Waldmann, 1978); and bone marrow chimeeras (von Boehmer *et al*, 1975a); have all been used to overcome the problems posed by real or potential alloreactivity.

The use of these techniques has given conflicting results. Studies involving *in situ* irradiation of T helper cells have been carried out by Pierce and Klinman (1975, 1976). Using the *in vitro* splenic fragment culture technique to examine antibody production by indvidual primary B cell clones in irradiated, reconstituted mice, it was shown that antibody formation was completely dependent on previous carrier priming of the host. The carrier-primed host could provide effective help to both syngeneic and allogeneic B cells *in vitro*; detailed analysis of the frequency of antibody-forming cell clones indicated it to be about 2/3 as great for allogeneic as syngeneic B cells. The allogeneic B cell clones, however, produced only small amounts of antibody, and exclusively of the IgM class, while large responses of IgG1 or IgG1 plus IgM classes were seen in syngeneic T-B cell combinations. This result thus suggested that B cell triggering *per se* is not dependent on T-B cell syngeny, but rather on antigen recognition by the T cell. Nonetheless, stimulation of IgG1 formation requires that T and B cells be syngeneic, either for effective help, or in preventing allogeneic inhibition. Subsequently, it was shown that the majority of secondary B cells could be stimulated to produce antibody of the IgG1 class by allogeneic and syngeneic T cells. Syngeneic T cells were slightly more efficient than allogeneic ones in enhancing the IgG1 response; nonetheless, specificity of stimulation by the allogeneic cells was suggested by the requirement that the T cells be carrier-primed, and that *in vitro* challenge had to be with the homologous hapten-carrier conjugate. This thus suggests a clear difference between primary and secondary B cells, particularly with respect to the H-2 requirements for activation to IgG antibody production. This result is difficult to reconcile with any of the models for adaptive differentiation of lymphoid cells, as homozygous nontolerant T cells should clearly be

unable to cooperate across H-2 differences. To account for this, Katz (1980) has postulated that small numbers of cells able to cooperate across H-2 differences may exist in normal animals, and be detectable only with particularly sensitive assay systems, such as the one used here. This explanation does not, however, address the very real difference between primary and secondary B cells.

Acute *in vivo* depletion of alloreactive cells by recirculation has also yielded evidence for unrestricted T - B cell cooperation in the primary *in vitro* response to heterologous erythrocytes (Heber-Katz and Wilson, 1975). Syngeneic and allogeneic T cells depleted of alloreactivity cooperated with B cells with equivalent efficiency, over a wide range of cell numbers; while allogeneic unfiltered cells showed quite different dose-response parameters. The apparent failure of high numbers of nontolerant cells to cooperate with B cells was attributed to suppression by T cell alloreactivity directed against B cell determinants; while the converse - increased efficiency of cooperation relative to syngeneic T cells seen at low cell doses -was ascribed to allogeneic enhancement. Finally, Swain *et al* (1977) obtained evidence indicating the possibility of cooperation across H-2 differences even for normal cells, if alloreactivity was avoided.

In contrast to the above results, indicating the possibility of allogeneic cooperation under at least some circumstances, many other experimental systems have shown strict H-2 restriction, even for tolerant cells. Thus, Kindred (1975) attempted to reconstitute nude mice with normal syngeneic, or neonatally tolerant allogeneic T cells. Syngeneic cells restored the SRBC response efficiently, while the use of allogeneic cells resulted in only a transient recovery of competence, followed by decline. This suggests that allogeneic cells are able to initiate a primary IgM response, but that its prolongation (and IgM -> IgG switch) requires syngeneic T - B cell interaction. In this respect, the results resemble those of Pierce and Klinman (1975).

Waldmann (1978) also used neonatally-tolerant cells in an attempt to circumvent normal H-2 restriction. I have already described his experiments indicating that the H-2 restriction seen for such tolerant cells is not a consequence of the priming environment; it follows from this that the *in vitro* T - B cell interaction is itself primarily restricted.

In vivo negative selection of homozygous cells by recirculation through irradiated semiallogeneic recipients, followed by *in vivo* assay, indicates that homozygous T cells

are fully H-2 restricted, despite the adequacy of depletion of alloreactive cells achieved (Sprent and von Boehmer, 1976). Strain *A* T cells cooperate only with *A* or (*A* x *B*)F_1 B cells, but not with *B* B cells. This failure of cooperation with allogeneic cells almost certainly does not reflect failure to reactivate the strain *A* helper cells in the absence of appropriate APC, as the *in vivo* assay is done in a 'neutral' (*A* x *B*)F_1 cooperating environment. Sprent *et al* (1980) have described further experiments, involving supplementation of the cooperating environment with appropriate APC, which also support this last conclusion.

Heterozygous T cells

In an earlier section, I have described the experimental evidence leading to the conclusion that (*A* x *B*)F_1 T cells normally exist as two discrete subpopulations, each restricted in helper cell induction to cooperation with APC of a single homozygous genotype. As a corollary of this, it has been observed that these restricted subpopulations also cooperate poorly with B cells allogeneic to the APC used in their induction. In addition to the rather good *in vivo* evidence indicating that this apparent H-2 restriction simply reflects a lack of appropriate APC in the ultimate cooperation step (Sprent, 1978c, d), *in vitro* experiments in which addition of appropriate APC failed to overcome restriction have also been described (Swierkosz *et al*, 1977; Yamashita and Shevach, 1978).

Directly opposed to such a tidy view, however, is the work of Singer *et al* (1979, 1980). In addition to the evidence outlined above, on the strict requirement for helper T cell - APC compatibility at the I-A subregion, these authors documented that T cells need not recognize the same H-2 determinants on APC and B cells; Thus, (*A* x *B*)F_1 T cells cooperate efficiently with *A* APC and *B* B cells (and the converse), either before or after priming *in situ*.

Stem cell chimaeras

von Boehmer *et al* (1975), Waldmann *et al* (1975, 1976), and Waldmann (1977) described successful physiological cooperative interactions between primed allogeneic T and B cells derived from two way bone marrow chimaeras *A* + *B* -> (*A* x *B*)F_1, *in vivo* and *in vitro*, respectively. Sado and Kamisaku (1975) obtained similar results for cells from one way bone marrow chimaeras, in the primary *in vivo* IgM response to SRBC. In

light of the work of Zinkernagel, several groups have extended their studies on genetic control of T - B cell interactions to include radiation chimaeras of various sorts.

Much of the work done with chimaeras which relates to T - B cell restriction, does so only as an consequence of T cell - APC restriction. Such studies show T cell cooperative preferences for B cells syngeneic with the APC used in *in vivo* priming (Waldmann *et al*, 1978; Kappler and Marrack, 1978; Sprent, 1978a; Waldmann, 1978), and suggest that T - B cell restriction is closely related to the T cell - macrophage restriction discussed above.

Nonetheless, evidence against this view has been presented by Singer *et al* (1979, 1980), and Hodes *et al* (1980). Thus (*A* x *B*)F_1 -> *A* chimaeric T cells were restricted to cooperation with *A* APC in the generation of a primary *in vitro* antibody response. Such T cells were, however, able to cooperate with *A*, *B*, or (*A* x *B*)F_1 B cells, and the failure of cooperation between F_1 -> *A* T cells, and *B* (APC + B cells) could be readily overcome by the addition of *A* APC. Similar results were obtained in *in vivo* adoptive transfer studies for a primary antibody response. These results suggest strongly that T cell recognition of B cell H-2 determinants, if it occurs at all, is not restricted to the MHC antigens seen on the APC; thus APC and B cell not be syngeneic.

Katz and his colleagues (1978) have done extensive studies on the cooperative preferences of T and B cells derived from a number of types of chimaeras; (*A* x *B*)F_1 -> F_1; *A* -> F_1; *B* -> F_1; F_1 -> *A*; and F_1 -> *B*. These authors claimed evidence for adaptive differentiation in both T and B cell populations, and in support of their hypothesis, adduced the evidence that F_1 -> F_1 chimaeric cells behaved indistinguishably from normal F_1 lymphocytes; while *A* -> F_1 or *B* -> F_1 chimaeric cells were identical to normal *A* or *B* cells respectively. Thus, *A* -> F_1 T cells primed *in situ* cooperated preferentially with *A* or (*A* x *B*)F_1 B cells. Cells from either F_1 -> *A* or F_1 -> *B* chimaeras showed aberrant cooperative preferences, cooperating efficiently only with cells syngeneic to the host environment in which differentiation occurred.

The finding of restricted cooperative preferences for parent -> F_1 chimaeric cells is quite compatible with most studies on the behaviour of such cells (Marusic *et al*, 1977; Waldmann *et al*, 1978), and with the hypothesis of Waldmann (1978) that this restriction is simply a manifestation of the presence of APC of only one haplotype in the

chimaeras.

These results can readily be interpreted in terms of an adaptive differentiation model (Katz, 1980), and are also compatible with the 'thymic learning' hypothesis of Zinkernagel *et al* (1978c).

More recently, however, Katz has reported data which are inconsistent with a predominant thymic role in determining restriction specificities, and which appear to correspond to the variable leakiness of restriction reported by some workers in the CTL system (Katz *et al*, 1979). These studies were carried out with thymus grafted F_1 -> ATxBM F_1 chimaeras, as used earlier by Waldmann *et al* (1979), and by Zinkernagel *et al* (1978c). Although some degree of preference for B cells syngeneic to the thymus graft was observed *in vivo*, the difference was of only marginal significance for IgG responses, and was not seen for IgE. Additionally, clear-cut results (ie. the presence or absence of help) such as were seen previously (Katz *et al*, 1978; Katz *et al*, 1973a, b) simply were not obtained. Technical points were apparently inadequate to account for the differences seen between F_1 -> parent and F_1 -> F_1 (ATxBM + parent thymus) chimaeras, and led to the notion that nonthymic tissues may have an important influence on T cell cooperative preferences.

This possibility was investigated further, in a series of experiments designed to control for the possible restricting influence of the APC present in the chimaeric environment (Katz *et al*, 1980b). Thus, T cells from various conventional and thymus-grafted chimaeras were adoptively primed in irradiated, thymectomized F_1 animals, and their helper activity assessed *in vivo*. It was found that, as predicted, parent -> F_1 chimaeras could be primed in an F_1 environment to cooperate across H-2 difference; this most likely reflects the provision of APC suitable to expand up both parentally-restricted subpopulations (Sprent *et al*, 1980). F_1 -> parent T cells primed *in situ* showed H-2 restriction, again as expected, but became capable of unrestricted interaction when subjected to further adoptive priming in irradiated F_1 recipients. Quite contrary to previous results (Waldmann *et al*, 1978), it also proved possible to prime neonatally tolerant spleen cells in an F_1 environment, to display non-H-2 restricted cooperation. T cells from thymic chimaeras, parent -> F_1 (ATx, parent BM + thymus) also were able to cooperate with MHC incompatible B cells after adoptive priming in an F_1

host. These findings do not lend themselves to interpretation in terms of a pure thymic influence scheme; in particular, that F_1 -> parent lymphocytes are capable of expressing an unrestricted cooperating phenotype. Indeed, they argue that the restriction patterns of fully-mature T cells are determined by predominantly extrathymic influences during peripheral differentiation, although the potential for allogeneic interactions is determined somewhat earlier in ontogeny.

The apparent restriction of *in situ* primed F_1 -> parent lymphocytes was thus termed a 'pseudorestriction', and ascribed to a process termed 'environmental restraint'. Environmental restraint, in turn, is a means by which the host environment can be nonpermissive for the expression of a particular cooperative phenotype. Thus, the (*A* x *B*)F_1 -> *A* chimaeric environment does not allow the expression of *B* restricted preferences by F_1 cells, but these can be revealed in an F_1 adoptive environment.

The concept of environmental restraint as described by Katz is sufficiently broad to encompass the above experimental observations within his framework for adaptive differentiation, based on the deletion of high affinity cells reactive against 'self' Cl molecules (Katz, 1980).

In addition to the evidence for adaptive differentiation and environmental restraint for T cells, Katz *et al* (1978) claimed to show a corresponding restriction in B cell function in F_1 -> parent chimaeras. This result was directly challenged by Sprent and Bruce (1979), who failed to find evidence for B cell and APC adaptive differentiation in F_1 -> parent chimaeras, showing instead that they behaved identically to normal F_1 cells. Given the substantial differences in experimental design, and particularly the sources of helper T cells used (parental by Katz; F_1 -> parental chimaeric by Sprent), the two systems are not directly comparable. The results nonetheless tend to suggest limits to the Katz hypothesis, even if they do not directly disprove it.

Antigen-presenting cell - B cell interactions

Much of the work described in this thesis relates to the possibility of regulation of the interaction between APC and B cells by the major histocompatibility complex. This phenomenon has not been previously described; yet there is some precedent for it in the literature.

Howie and Feldmann (1978) described the apparent expression of *Ir* genes for (T,G)-A--L at the macrophage - B cell interaction. Using a T cell derived helper factor specific for (T,G)-A--L, and B cells derived from (R x NR)F_1 animals, they showed that the *in vitro* antibody response could be restored only by R or (R x NR) F_1, but not by NR APC. This result thus makes it unlikely that APC function is purely passive, for example, concentrating helper factor for effective presentation to the B cell, and the authors suggest the possibility of genetically-restricted APC - B cell interactions, analogous to those between APC and T cells (Erb *et al*, 1975a - d).

Gorczynski *et al* (1980) presented evidence for the induction of H-2 restriction in normal F_1 B cells, by priming in an irradiated parental environment. These cells could subsequently be activated *in vitro* by antigen on APC or activated T cells syngeneic to the priming host, in an assay system in which helper T cell function was replaced by suboptimal quantities of LPS. This restriction could not, however, be shown in an *in vivo* T cell dependent assay.

A strict macrophage requirement in the *in vitro* response to the thymus-independent antigen TNP-Ficoll was reported by Boswell *et al* (1980c), and it was further shown that only viable TNP-Ficoll pulsed spleen adherent cells could present the antigen to B cells. Similar to the results of Howie and Feldmann (1978), this suggests an active role for the APC in the presentation of antigen to the B cell, and also the potential for H-2 restriction of this interaction. Subsequent studies by the same group (Boswell *et al*, 1980a, b) indicated that APC mediated antigen presentation activates only a Lyb 5+ B cell subpopulation. Treatment of B cells with anti-Lyb 5 antiserum and complement eliminated the *in vitro* responses to the thymus dependent antigen, TNP-KLH, and to the TI-2 antigen, TNP-Ficoll. The responses to free TNP-LPS and TNP-*Brucella abortus*, both TI-1 antigens, were unaffected. TI-1 antigen pulsed accessory cells could, however, activate only Lyb 5+ B cells, suggesting that this subpopulation could be activated only by signals delivered via accessory cells. The CBA/N mouse, which lacks the Lyb 5+ cell subpopulation, was used to verify this prediction. It was shown that CBA/N mice were unable to give primary *in vitro* B cell responses to TNP-KLH, despite the presence of fully functional APC and T cells. APC were also shown to be fully competent to present TNP-Ficoll and TNP-BA to syngeneic normal B cells, but

not to CBA/N B cells, while free TNP-BA in high concentrations could activate CBA/N B cells.

This suggests a deficit in the interaction between accessory cells and B cell for antigens of all classes in the CBA/N mouse. All of the immunological findings in this strain can be explained on the basis that activation of the Lyb 5+ cell population is dependent on signals delivered by accessory cells. Although this does not prove genetic restriction of this interaction, the situation described here resembles certain findings in H-2 restricted cell interactions.

II. Development of research program

The studies described in this thesis fall logically into several discrete and self-contained units, although all are related to the influence of the major histocompatibility complex on cellular interactions. They are not presented in a chronological order, but rather in a sequence which illustrates the conceptual progression of this work.

We wished in the first instance to approach the question of cellular expression of immune response or *Ir* genes. Initially, we chose to concentrate on the interaction between APC and primed T cell, and obtained results equivalent to those of Rosenthal and Shevach (1973) in the guinea pig, showing an apparent antigen specific defect in APC function from nonresponder animals to TNP-18. Yano *et al* (1978) independently obtained similar results for the random terpolymer GAT. This assay system requires the use of heterozygous (R x NR)F_1 T cells and either R or NR APC, to eliminate possible allogeneic effects, and thus is limited to evaluation of *Ir* gene expression by the APC.

In order to allow us to extend these studies to other possible cellular sites of *Ir* gene expression, including the T cell, it was necessary for us to devise alternative models in which the complication of alloreactivity associated with the use of parental cells can be avoided. We chose to use parent -> F_1 stem cell chimaeras, a fortunate decision, although our initial reasons for doing so were largely incorrect. We used foetal liver for reconstitution rather than the more usual bone marrow. In other respects, our protocol followed that of Sprent *et al* (1975). Subsequently, it was somewhat modified as the importance of thorough depletion of T cells from the donor stem cells became appreciated, and appears schematically in this form in Figure 1. Stem cell chimaeras were also preferred over other means of tolerance induction, as we assumed at the time that the tolerance to MHC antigens attained by long residence in a semiallogeneic host is itself sufficient to allow cooperation across H-2 differences, and that the APC function in irradiated reconstituted animals is derived from the host, rather than from the stem cell donor. If these assumptions are made, it follows that it should be possible to prime parental cells in the context of host derived (F_1) APC, and thus to look directly for *Ir* gene control of T cell function in nonresponder *versus* responder cells. To verify this approach, we initially looked at H-2 control of the T cell - APC interaction with respect to the non-*Ir* gene controlled antigen, PPD, after priming with CFA containing *M.*

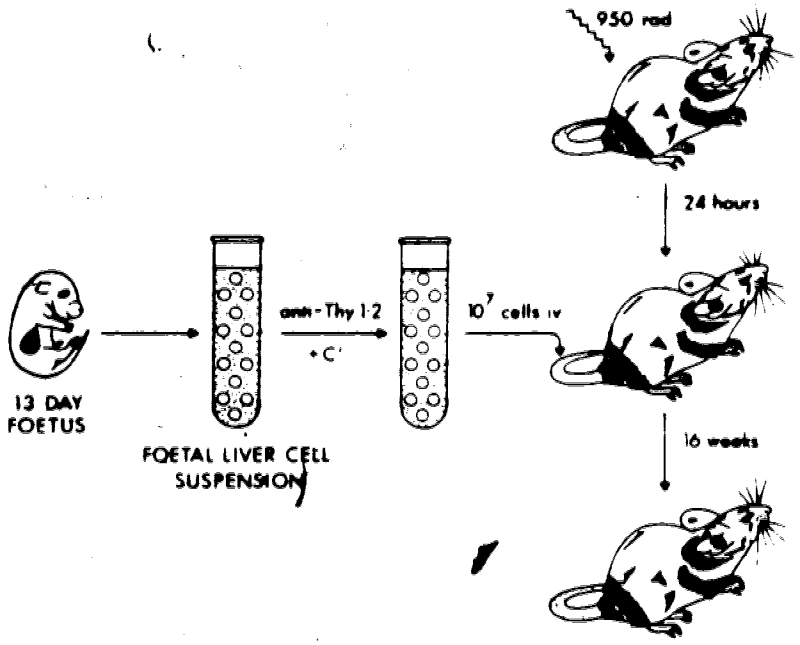


Figure 1. Protocol for the preparation of foetal liver chimeras. Modified from Sprent *et al*, 1975). Supralethally irradiated (C3H.OH x C3H.A)F_1 animals are reconstituted after 24 hours with 10^7 anti-Thy 1.2 + C' treated 13 day foetal liver cells from C3H.OH or C3H.A donors.

tuberculosis. To our surprise, parent -> F_1 chimaeric T cells were restricted to cooperation with syngeneic APC. Although this prevented us from using the system to dissect out the cellular expression of *Ir* genes, it provided useful information on H-2 restriction. The results were quite compatible with the hypothesis of Zinkernagel *et al* (1978a,b) on dual thymus - macrophage control of H-2 restriction specificities, and with subsequent evidence that the APC in the chimaeras are derived from the reconstituting donor cells.

To overcome the problems encountered with the previous approach, we turned to two way stem cell chimaeras $A + B$ -> $(A \times B)F_1$, described by von Boehmer *et al* (1975b). In these animals, differentiation through an F_1 thymus, and the presence of APC of both parental haplotypes would be predicted to allow unrestricted T cell cooperation with APC in the response to non-*Ir* controlled antigens. For antigens under *Ir* gene control, conditions would exist so that NR T cells could at least theoretically be primed in the presence of responder APC. We were, however, unable to show H-2 unrestricted behaviour for T cells primed *in situ* with either KLH or CFA, both non-*Ir* gene controlled antigens. The reasons for our inability to obtain results compatible with the Zinkernagel hypothesis still elude us, as other groups have shown two-way chimaeric cells to be unrestricted in similar (Schwartz *et al*, 1979; Longo and Schwartz, 1981) and different experimental systems. Results analogous to ours have been reported by Erb *et al* (1978, 1979) for unprimed T cells from two-way bone marrow chimaeras.

Substantial evidence has been obtained that the cooperative properties of cells obtained from chimaeras may be strongly influenced by the adequacy of elimination of alloreactive cells from the cell populations used for reconstitution. We felt that the best way to examine this point was by the use of tetraparental chimaeras derived by *in vitro* fusion of eight cell embryos of H-2 incompatible strains. Under these conditions, chimaerism is established long before the emergence of the immune system, and thus any influence of the donor environment on the cooperative preferences of the mature cells should be avoided. The validity of this approach is suggested by studies with a limited number of tetraparental mice indicating that the T cell cooperative preferences can in fact be shifted away from self. In view of this, we feel that our assay system is sufficiently sensitive to detect haplotype preferences existing prior to priming, which

may be obscured by the priming conditions used by other workers.

The other major series of experiments was similarly intended to allow us to look at *Ir* gene expression between T cells, APC, and B cells using an *in vivo* adoptive transfer system for the antibody response. To obviate interference from allogeneic effects, the cooperating T and B cells were derived from parent -> F_1 chimaeras. Calibration experiments carried out with non- *Ir* gene controlled antigens indicated a then-unexpected H-2 restriction between APC and B cells. In view of this, and the results previously obtained in the T cell proliferation assay, we decided to use this model for the study of H-2 restriction. B cell - APC restriction was shown not to be due to suppression mediated by allogeneic effects between the irradiated host and the transferred T and B cells. Similar results have been claimed previously for thymus independent antigens, and we believe we are able to show this restriction for thymus dependent antigens only as the chimaeric helper T cells behave in an H-2 unrestricted fashion under our particular experimental conditions.

III. Materials and Methods

The experimental procedures described in this section are common to many of the studies in this thesis. Specific variations on these, and techniques restricted to particular experiments are given in the appropriate sections.

Mice: Inbred mice of strains Balb/cCr (H-2*d*), C3H.HeJ (H-2*k*), C3H.Sw/Sn (H-2*b*), C3H.OH (H-2*o2*), and C3H.A(H-2*a*), and F_1 hybrids (C3H.HeJ x C3H.Sw/Sn) and (C3H.OH x C3H.A) were obtained from the Small Animal Breeding Program, The University of Alberta, Edmonton, Alberta. Vaginally plugged female mice of the above and the outbred ICR strains were provided by the same source. Haplotypes are shown schematically in Table 1.

Animals were housed 4 - 6 per cage with free access to food and acidified chlorinated water. Irradiated mice were given gentamicin sulphate (Schering), 8 ug subcutaneously and carbenicillin (Ayerst), 2 mg intravenously per eight hours for the first two days after irradiation. Mice were maintained on water containing tetracycline (Tetracyn) 10 mg per 250 ml of water.

Irradiation: Mice and cells were exposed to ^{137}Cs irradiation at a dose rate of 98-96 rad per minute from a 'Gamma Cell 40' (Atomic Energy of Canada Limited, Ottawa, Ontario. The radiation doses used in particular studies are specified in the appropriate sections.

Media: Leibowitz L-15 medium (Grand Island Biological Company of Canada, Calgary, Alberta) supplemented with 10% foetal calf serum (GIBCO) was used for the preparation of cell suspensions, nylon wool filtration for T cell enrichment, and plastic adherence for depletion of adherent cells.

Cell preparations: Single cell suspensions were prepared from foetal liver by mincing with iris scissors, and passing the fragments through a metal sieve (pore size 0.2 mm). Clumps were broken by gently aspirating the suspension several times through a Pasteur pipette.

Spleen and lymph nodes were teased apart with sterile 23 gauge syringe needles, and clumps allowed to settle out under gravity for five minutes.

Antiserum treatment of cell suspensions: Except as otherwise stated, cells were treated with antibody using a standard two stage cytotoxicity assay.

Table 1: H-2 haplotypes of mouse strains, and regions of the H-2 complex recognized by antisera used in these studies.

Strain	Abbrev.	K	A	B	J	E	C	S	G	D
DBA/2J	H-2*d*	d	d	d	d	d	d	d	d	d
Balb/cCr	H-2*d*	d	d	d	d	d	d	d	d	d
C3H.HeJ	H-2*k*	k	k	k	k	k	k	k	k	k
C3H.SwSn	H-2*b*	b	b	b	b	b	b	b	b	b
C3H.OH	H-2*o2*	d	d	d	d	d	d	d	d	k
C3H.A	H-2*a*	k	k	k	k	k	d	d	d	d
(A.TH x C3H.OL) anti-C3H		k								
(A.TL x B10.A) anti-B10.BR										k
(B10 x A) anti-B10.D2		d	d	d	d	d				
(B10.A(2R) x C3H.Sw) anti-C3H							k	k	?	k
(B10.A(2R) x A.CA) anti-HTG		d	d	d	d	d				

Cells were suspended at 10^7 per ml in RPMI-1640 medium (GIBCO) buffered with 25 mM HEPES (Calbiochem, San Diego, CA) and 3.5 g/l $NaHCO_3$ and supplemented with 0.3% Bovine Serum Albumin (Sigma Chemical Company, St. Louis, MO), containing an appropriate dilution of antiserum. After incubation on ice for 45 minutes, the cells were pelleted, washed, and resuspended in the same volume of medium containing diluted complement. The cells were then incubated at 37°C for 45 minutes, and either counted directly with eosin, or washed and counted.

Antiserum and complement sources are specified in the appropriate sections. The H-2 specificities recognized by the different antisera appear in Table 1.

Cell counts: Cell counts were performed with a modified Neubauer haemocytometer (American Optical, Inc., Buffalo, NY), and viability determined by the eosin dye exclusion test of Hanks and Wallace (1958).

Cell injections: Mice were kept in a 37°C warm room for 30-45 minutes to dilate the tail veins. Cell suspensions were made up in L-15 medium without serum, and injected into the lateral tail vein with a 26 ga. x 1/2 inch needle.

Nylon wool filtration for T cell purification: Nylon wool (Fenwal Laboratories Inc.) was washed by autoclaving once with 0.1 N HCl, followed by six cycles of autoclaving with glass double distilled water, and air dried. Five, ten, and twenty millilitre columns were prepared by packing disposable syringe barrels with 0.3, 0.6, and 1.2 grams of dry nylon wool, respectively, and autoclaved.

The filtration technique employed was that of Julius *et al* (1973), except that L-15 + 10% FCS was used in place of Dulbecco's PBS. The packed columns were washed with medium, and equilibrated with medium at 37°C for 45 minutes. Two to three x 10^8 cells in 0.5 ml of warm medium were applied to a 10 ml column, and washed in with a further 0.5 ml of medium. After incubation at 37°C for approximately one hour, nonadherent cells were eluted with 50 ml of warm L-15 + 10% FCS. These preparations were 85-90% lysed on treatment with anti-Thy 1.2 antibody and complement.

Purification of B cells: Single cell suspensions prepared from spleen as described above were treated twice with anti-Thy 1.2 antibody and complement as described above. The remaining cells were suspended at 10^7 cells per ml in L-15 + 10% FCS, and 10 ml placed in Corning 100 x 15 mm tissue culture dishes (Corning, Corning

NY). After incubation for 1 hr at 37°C, the nonadherent cells were dislodged by gentle shaking, and transferred to new dishes. This procedure was repeated three times. The plastic nonadherent cells were recovered and washed twice before injection.

IV. MHC restriction of the T cell - antigen presenting cell interaction. I. Normal and one way foetal liver chimaeric cells

A. Introduction

The ability of histoincompatible cells to collaborate efficiently in the development of an immune response remains in dispute. Early work in the mouse suggested that T - B cell interactions are under the control of the major histocompatibility complex, and are allogeneically restricted: primed T cells could not cooperate across an I-region barrier (Katz *et al*, 1973b). Similar conclusions were reported for T cell - macrophage interactions in the guinea pig, using a T cell proliferation assay (Rosenthal and Shevach, 1973). In retrospect, these early studies on cell cooperation are subject to the criticism that MLR-like or allogeneic effects arising from the mixing of histoincompatible cells were not excluded (Gorczynski *et al*, 1976).

The two way bone marrow chimeric mouse, in which all lymphoid cells are mutually-tolerant, was used in an attempt to circumvent this problem. It was possible to show effective cooperation between histoincompatible T and B cells from these animals in the generation of an antibody response, and it was claimed that the tolerance to alloantigens achieved in these animals was sufficient to allow interaction (von Boehmer *et al*, 1975a). This subsequently gave rise to the concept that cells could learn to cooperate during ontogeny through a process of 'adaptive differentiation' (Katz, 1977b). On the basis of studies on the generation of T helper cells for antibody, and cytotoxic T cells directed against virus-infected targets, this theory has been extended and it has been proposed that the thymus in conjunction with the peripheral lymphoreticular system determines the H-2 restriction specificities ultimately possessed by the mature cell (Zinkernagel *et al*, 1978a; Waldmann *et al*, 1979). By contrast, the macrophage - T cell interaction has been less well studied. Most work has examined the primary generation of T helper cells; conflicting results have been obtained (Pierce *et al*, 1976; Erb and Feldmann, 1975a). It has recently been shown that allogeneic restriction applies to sensitized nonchimeric cells, but not to cells from two-way chimaeras in the adoptive transfer of delayed-type hypersensitivity to naive recipients (Vadas *et al*, 1977).

The expression of immune response or *Ir* genes at the level of the macrophage was first described in the guinea pig for the PLL system (Shevach and Rosenthal, 1973), and subsequently confirmed in the mouse for the synthetic copolymer GAT (Yano *et al.*, 1978), and the *Ir*-gene controlled determinants of insulin (Rosenthal, 1978). It is clear that there is considerable similarity between allogeneic restriction and *Ir* gene expression by the macrophage, and that these two events may represent different manifestations of the same general phenomenon (Miller and Vadas, 1977). In view of this, we have used the T cell proliferation assay to examine the genetic constraints on the macrophage – T cell interaction for both multideterminant antigens and simple synthetic peptides under *Ir* gene control. In the present paper, we show allogeneic restriction in the response to PPD at the macrophage level for T cells from one-way foetal liver chimaeras, for macrophage associated antigen. Interestingly, when antigen was present in soluble form, restriction could not be shown for either normal or chimaeric T cells. Rosenwasser and Rosenthal (1978) have reported a similar finding for nonchimaeric T cells and multideterminant antigens, although their discussion did not exclude possible influence of nonspecific allogeneic effects.

We have continued our studies with synthetic peptide antigens of defined primary and secondary structure (Singh *et al.*, 1978), the response to which is under H-2 linked *Ir* gene control. We show here an antigen specific macrophage defect in nonresponder strains to the antigen [Glu-Tyr-Lys(TNP)(Glu-Tyr-Ala)$_3$] (TNP-18); although other levels of *Ir* gene control cannot be excluded. It was found, further, that both responder and nonresponder macrophages would collaborate efficiently with free TNP-18 in supporting a proliferative response. Supernatants from cultured macrophages were capable of replacing macrophage function under these conditions.

B. Materials and Methods

Antigens: Purified protein derivative of tuberculin (PPD) was purchased from Connaught Medical Research Laboratories, Willowdale, Ontario. The synthetic peptide antigen [Glu-Tyr-Lys(TNP)(Glu-Tyr-Ala)$_3$] (TNP-18) was synthesized as described previously (Barton *et al.*, 1977).

Media: RPMI-1640 medium (GIBCO) buffered with bicarbonate and 25 mM HEPES (Calbiochem) without added serum was used for incubation of macrophages with antigen, and RPMI-1640 medium with bicarbonate buffer (3.5 g $NaHCO_3$ per litre) supplemented with 10% heat inactivated normal human serum and gentamicin sulphate (50 ug per ml) was used for cell cultures.

Cell preparations: Peritoneal exudate cells (PEC) were harvested by lavage of the peritoneal cavity of normal mice with sterile Puck's saline. The cells were washed twice and irradiated with 1500 rad from a ^{137}Cs source.

Antigen-pulsed APC ('bound antigen') were prepared by incubating APC at a density of 10^7 cells per ml with PPD to a final concentration of 200 ug per ml for 1 hour at 37° C. The cells were subsequently washed twice with washing medium, and once with RPMI-1640 without serum, before being resuspended in culture medium.

PEC supernatants were prepared as described by Feldmann for Nonspecific Macrophage Factor (NMF) (Erb and Feldmann, 1975b). (Figure 2). Adherent PEC were treated with rabbit anti-mouse brain antiserum (Cedar Lane Laboratories Limited, London, Ontario) and complement (Cedar Lane 'LO-TOX M'), and rabbit anti-mouse Ig antiserum and complement, irradiated with 1500 rad of gamma irradiation, and washed. The cells were then incubated for four days in culture medium, and the supernatants harvested and sterilized by passage through a 0.45 um Millipore 'Swinnex' filter (Millipore Corp., Bedford, MA.). Supernatants were stored at -70°C until use.

Chimaeras: (C3H.Sw/Sn x C3H.HeJ)F_1 and (C3H.OH x C3H.A)F_1 mice were lethally irradiated (850 rad) and reconstituted within 6 hours by the intravenous injection of 10^7 15-day foetal liver cells from the appropriate parental strain. Survival varied between 65% and 90%, and survivors were >90% reconstituted with cells of donor origin, as assessed by dye-exclusion testing with cytotoxic antisera, using a two stage procedure. Alloantisera were kindly provided by Dr. T.L. Delovitch of the University of Toronto.

Immunizations: Mice were immunized by injection into both hind footpads of a total of 0.1 ml of an emulsion consisting of equal parts of Complete Freund's Adjuvant (0.5 mg per ml heat killed *M. tuberculosis* H37Ra) (Difco Laboratories, Detroit, MI.), and saline. For TNP-18 priming, antigen was included in the emulsion at a final concentration of 30 ug per 0.1 ml. The popliteal lymph nodes were removed at between 7 and 9 days

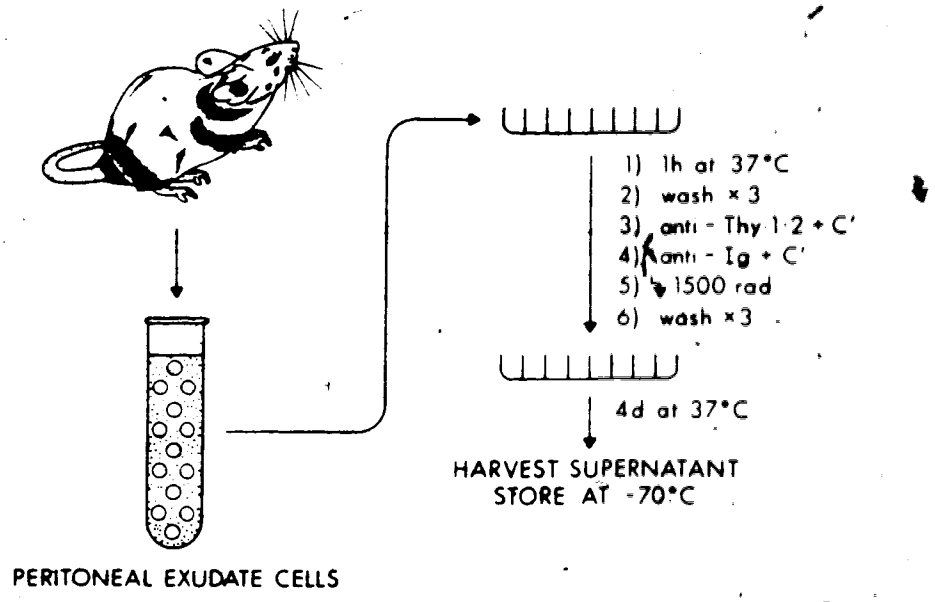


Figure 2: Protocol for the preparation of Nonspecific Macrophage Factor (after Erb and Feldmann, 1975b) Adherent peritoneal exudate cells are treated with anti-Thy 1.2 + C' and anti-Ig + C', irradiated with 1500 rad from a ^{137}Cs source, and cultured for 4 days at 37°C. The culture supernatant contains Nonspecific Macrophage Factor (NMF) activity.

after priming, and cell suspensions prepared as described above.

Cell culture technique: The T cell proliferation assay of Lee and co-workers (1979) was used (Figure 3). Lymphocytes and APC were cultured together in 200 microliters of culture medium in flat-bottomed sterile microtiter plates (Linbro FB-96-TC, Flow Laboratories), for 120 hours at 37° C in an atmosphere of 10% CO_2 in air. All cultures were carried out in triplicate. A range of lymphocyte and APC numbers was generally set up; in most instances, optimal responses were obtained with 4 x 10^5 lymphocytes, and 3 x 10^4 macrophages per well or 10% supernatant. Antigen-pulsed APC were added as appropriate. Twenty-four hours before harvesting, 0.7 microcurie of [methyl-^{3}H]thymidine (specific activity 2.0 Ci per mmol)(Amersham Corp., Oakville, Ontario) was added to each well. Cells were harvested onto glass filters with a Titertek semiautomated multiple sample collector (Flow Laboratories, Inglewood, CA). Thymidine incorporation was determined by liquid scintillation spectrometry, and the results reported as mean ± standard error of the triplicate.

C. Results

The T cell proliferation assay used in these studies has been previously shown to have an absolute macrophage dependence for multideterminant antigens such as PPD (Lee *et al*, 1979). In light of this observation, we investigated the ability of antigen-pulsed syngeneic or allogeneic macrophages to stimulate the proliferative response of primed, macrophage-depleted lymph node cells.

Macrophage haplotype and restoration of the T cell proliferative response

Two combinations of congenic resistant mice, on the C3H background, were used: C3H.Sw/Sn (H-2*b*) and C3H.HeJ (H-2*k*), and the intra-H-2 recombinants C3H.OH (H-2*02*) and C3H.A (H-2*a*).

The proliferative response of T cells from CFA primed C3H.Sw/Sn or C3H.HeJ mice was restored equally well by PPD-pulsed macrophages from either parental type; that is, allogeneic restriction was not observed. As expected, (C3H.Sw/Sn x C3H.HeJ)F_1 T cells cooperated with macrophages of either parent. To exclude the possibility that this apparent lack of restriction seen with parental T cells represented an artifactual allogeneic effect due to mixing of fully histoincompatible T cells and APC, T cells from

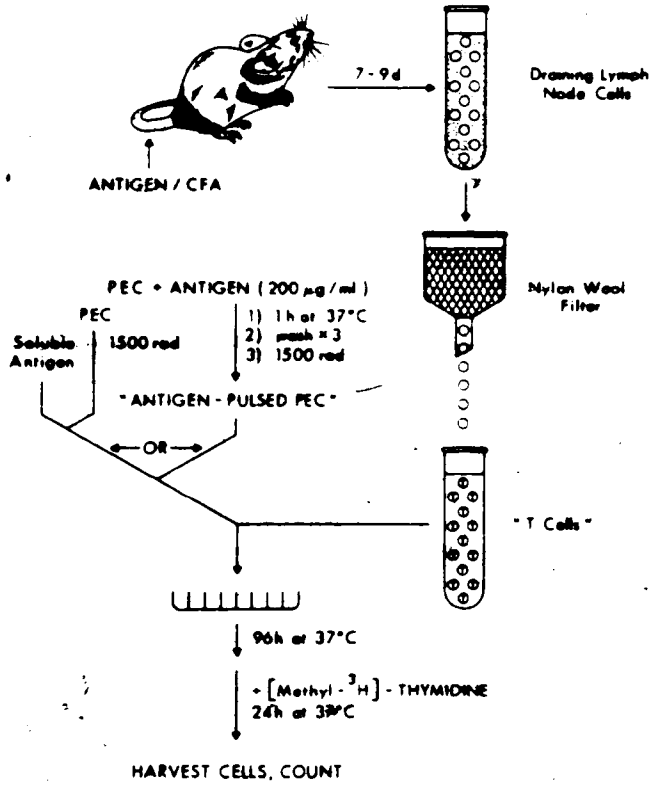


Figure 3: Protocol for *in vitro* T cell proliferation assay (Lee *et al*, 1979) Nylon wool filtered ('T') cells from the popliteal lymph nodes of mice primed 7–9 days previously with antigen/CFA in both hind foot-pads are cultured for 120 hours with antigen-pulsed antigen presenting cells (APC) or APC plus soluble antigen. [methyl-^{3}H]-thymidine is added for the last 24 hours of culture, and cells are harvested and counted for incorporation of label.

foetal liver chimaeras parent -> F_1 were used in some studies. The degree of tolerance achieved in these chimaeras was assessed by the inability of chimaeric lymph node cells to respond in mixed lymphocyte culture. Under these conditions, allogeneic restriction was observed; T cells from the chimaeras recognized antigen only in association with syngeneic APC (Table 2). These experiments have been repeated using the intra-H-2 recombinants C3H.OH and C3H.A, and parent -> F_1 chimaeras of those strains, again with PPD as the antigen. Strong MLR-like effects masked the antigen-specific response of nonchimaeric T cells in this instance, and consequently no conclusions can be drawn regarding the ability of nontolerant T cells and APC to cooperate. It was found, however, that the chimaeric T cells collaborated effectively only with syngeneic and not with allogeneic macrophages (Table 3). We have obtained comparable findings with other antigens such as KLH.

T cell proliferation was markedly stimulated by free antigen in the presence of macrophages, irrespective of the macrophage haplotype. Syngeneic and allogeneic macrophages supported proliferation equally well under these circumstances, and the chimaeric or nonchimaeric origin of the T cells was also apparently irrelevant (Tables 2, 4). The response was nonetheless macrophage dependent, as free antigen alone was not stimulatory, while the proliferative response varied directly with antigen concentration when the macrophage number was kept constant in culture (Figure 4). The dependence of the proliferative response on the number of APC per culture, for soluble and APC-associated antigen was similarly determined (Figure 5).

Genetic restriction at the macrophage level in the response to TNP-18

The synthetic peptide antigen TNP-18 [Glu-Tyr-Lys(TNP)(Glu-Tyr-Ala)$_3$] differs from the random copolymers previously used in having defined primary and secondary structures. The response to this antigen has been shown to be under H-2 linked *Ir* gene control, and responder and nonresponder strains have been identified. The intra-H-2 recombinants C3H.OH (I-A*d*) (responder) and C3H.A (I-A*k*) (nonresponder), and the (C3H.OH x C3H.A)F_1 (responder) were used in these studies. Nonresponders to this antigen are absolute nonrsponders, in that no immunoglobulin of any class is produced, and DTH cannot be elicited. Suppressor cells are absent in the TNP-18 system (B. Singh, unpublished).

Table 2 Reconstitution of the PPD proliferative response of normal and chimaeric cells by syngeneic and allogeneic macrophages.

T cells[1]	APC[2]	Response (cpm ± SE) No Ag	Free Ag[3]	bound Ag[4]
C3H.HeJ unfractionated		5170 ± 2220	107230 ± 13900	
C3H.HeJ NWF	C3H.HeJ	10190 ± 4160	105180 ± 4160	56180 ± 9900
	C3H.Sw/Sn	6500 ± 5440	103100 ± 3850	56120 ± 2760
C3H.Sw/Sn unfractionated		930 ± 70	79260 ± 5530	
C3H.Sw/Sn NWF	C3H.HeJ	5080 ± 1140	105510 ± 8160	112500 ± 12580
	C3H.Sw/Sn	15460 ± 6670	100660 ± 6630	86940 ± 2600
F_1 unfractionated		3860 ± 2460	115850 ± 7840	
F_1 NWF	C3H.HeJ	5050 ± 1090	99730 ± 5700	115740 ± 4640
	C3H.Sw/Sn	16830 ± 3300	79300 ± 9050	115740 ± 4640
C3H.HeJ -> F_1 unfractionated		1450 ± 160	54860 ± 200	
C3H.HeJ -> F_1 NWF	C3H.HeJ	2080 ± 1090	35000 ± 2100	33500 ± 3830
	C3H.Sw/Sn	5700 ± 1220	35400 ± 5510	9640 ± 630
C3H.Sw/Sn -> F_1 unfractionated		2880 ± 920	95640 ± 1250	
C3H.Sw/Sn -> F_1 NWF	C3H.HeJ	760 ± 310	40410 ± 5440	3700 ± 660
	C3H.Sw/Sn	1210 ± 320	47640 ± 2570	13560 ± 1220

[1] T cell donors were primed 8 days previously in both hind footpads with an emulsion of equal parts of Complete Freund's Adjuvant and saline. Preparation of cell suspensions and culture conditions is described in *Materials and Methods*. 4 x 10^5 cells were used in each culture.

[2] Irradiated (1500 rad) cells obtained from peritoneal washings of normal mice. Results are shown only for the optimal APC number (3 x 10^4 per culture).

[3] Purified Protein Derivative of tuberculin (PPD) added to a final concentration of 100 ug/ml in culture.

[4] Macrophages at 10^7 per ml incubated with PPD (200 ug/ml) for 1 h at 37°C, washed x 3 with medium, and diluted to the appropriate final concentration.

Table 3: Allogeneic restriction of the lymphocyte – APC interaction for PPD, in the T cell proliferation assay.

T cells[1]	APC[3]	Response (cpm ± SE) No Ag	Free Ag[3]	bound Ag[4]
C3H.A unfractionated		5130 ± 230	83550 ± 7190	
C3H.A NWF	C3H.A	3698 ± 1910		40531 ± 3570
	C3H.OH	53902 ± 8520		48475 ± 1690
C3H.OH unfractionated		14260 ± 1810	63170 ± 4730	
C3H.OH NWF	C3H.A	25950 ± 2200		29930 ± 5060
	C3H.OH	1920 ± 610		18020 ± 4510
C3H.A -> F_1 unfractionated		7960 ± 1690	67370 ± 4170	
C3H.A -> F_1 NWF	C3H.A	1640 ± 120		28760 ± 5420
	C3H.OH	540 ± 60		1120 ± 160
C3H.OH -> F_1 unfractionated		5070 ± 548	67270 ± 1780	
C3H.OH -> F_1 NWF	C3H.A	4790 ± 1120		5440 ± 670
	C3H.OH	2930 ± 140		40240 ± 3740

[1-4] The remarks of Table 2 *vid. sup.* apply to these experiments.

[3] The optimal macrophage number was determined to be 10^4 cells per culture in these studies.

Table 4: The macrophage dependent T cell proliferative response does not shown allogeneic restriction for antigen free in culture.

T cells[1]	APC[2]	Response (cpm ± SE) No Ag	Free Ag[3]	bound Ag[4]
C3H.OH unfractionated		4030 ± 590	70500 ± 3970	
C3H.OH NWF	C3H.A	2838 ± 391	31833 ± 4141	8758 ± 1840
	C3H.OH	227 ± 39	35588 ± 5516	17049 ± 3798
C3H.OH -> F_1 unfractionated		290 ± 120	43320 ± 2690	
C3H.OH -> F_1 NWF	C3H.A	591 ± 456	71025 ± 4343	3164 ± 763
	C3H.OH	384 ± 16	55611 ± 5017	47506 ± 4376
F_1 unfractionated		1270 ± 270	56940 ± 6010	
F_1 NWF	C3H.A	396 ± 162	50972 ± 3324	20450 ± 2586
	C3H.OH	209 ± 83	65472 ± 2938	39711 ± 3194

1-4 The remarks of Table 2 *vid. sup.* apply to these experiments.

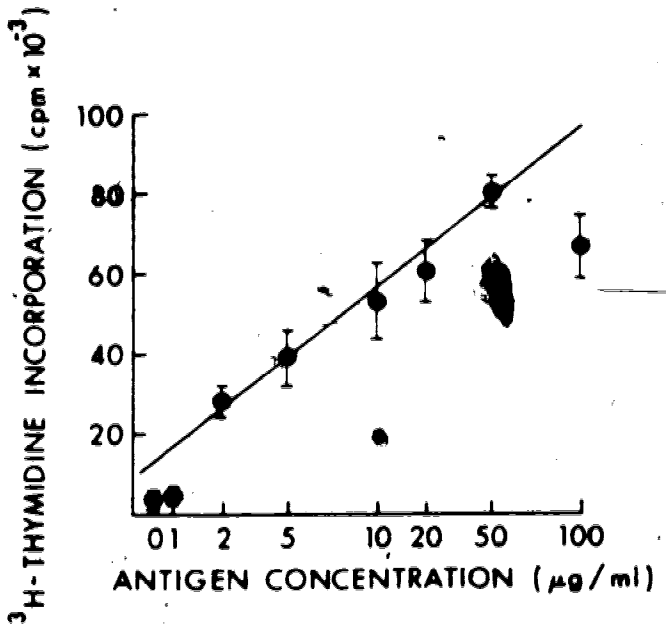


Figure 4: The proliferative response of primed lymph node T cells is a direct function of the concentration of antigen present in culture. The T cell proliferation assay was carried out as described above, with 4 x 10^5 CFA-primed lymph node cells and 3 x 10^4 PEC per well. The PPD concentration was varied over the range 0 - 100 ug/ml.

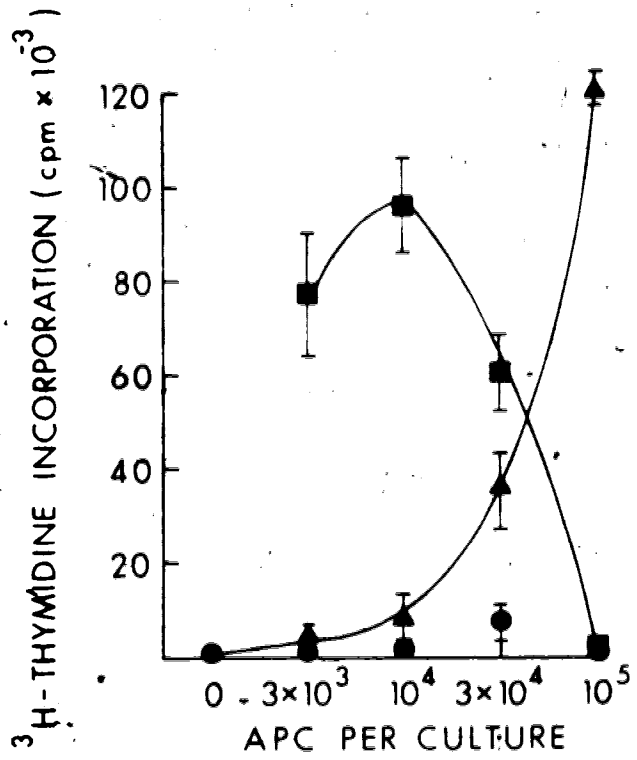


Figure 5: The T cell proliferative response as a function of the number of APC added in culture, for free and APC-associated antigen. The T cell proliferation assay used 4 x 10^5 CFA-primed, NWF LNC per well, with various numbers of antigen pulsed PEC (▲) or normal PEC (■). The antigen concentration for the latter was kept constant at 50 ug/ml.

The proliferative response of primed (C3H.OH x C3H.A)F_1 T cells to TNP-18 was restored only by antigen associated with responder (C3H.OH) or F_1 macrophages; nonresponder (C3H.A) APC were clearly ineffective (Table 5). By contrast, the response of the same T cells to the non-*Ir* gene controlled antigen PPD was restored by both C3H.OH and C3H.A macrophages, suggesting that the defect in C3H.A macrophages is antigen specific.

It was observed that the proliferative response to *free* TNP-18 was restored equally well by responder and nonresponder macrophages. The experiments described in the next section were set up to examine this pont in more detail.

Macrophage culture supernatants are not I-region restricted

It has been known for some time that the supernatants of macrophages cultured without antigen are able to replace macrophage function nonspecifically in the *in vitro* generation of T helper cells (Calderon *et al*, 1975). In light of this, and in view of our results suggesting that macrophages can function nonspecifically in the presence of free antigen, the ability of supernatants to replace macrophage function in the T cell proliferation assay was investigated. Supernatants were prepared from responder and nonresponder macrophages and assayed as described in *Materials and Methods*; they were found to act synergistically with both the *Ir* gene controlled antigen, TNP-18, and PPD, regardless of their origin from responder or nonresponder APC (Tables 5, 6). The proliferative response obtained was a direct function of factor concentration over the range 0.1% to 10% (Figure 6). TNP-18 in the absence of supernatant stimulated some degree of T cell proliferation. The reasons for this are not clear, but this result may represent direct triggering of lymphocytes by antigen, perhaps a reflection of the high concentration of soluble antigen used (200 ug per ml): the effect was less at lower concentrations. The possibility that supernatant acts in cooperation with residual and necessarily syngeneic macrophages in the T cell proliferation seems unlikely, as repeated nylon wool filtration of the lymph node cells did not abolish the effect of supernatant on the PPD proliferative response, while markedly abrogating the Con A response of the same cells. (Table 6). These data suggest that supernatants can replace at least some macrophage functions in the proliferation assay. Although their mechanism of action is unknown, supernatants may amplify an established proliferative response.

Table 5: Macrophage level *Ir* gene effects in the proliferative response of primed T cells to the synthetic peptide antigen TNP-18.

T cells[1]	APC or S/N[2]	Response (cpm ± SE) No Ag	Free Ag[3]	bound Ag[4]
(C3H.OH x C3H.A)F_1 unfr.		1440 ± 120	50800 ± 7990	
(C3H.OH x C3H.A)F_1 NWF	3 x 10^4 C3H.A	8600 ± 710	49160 ± 7260	10350 ± 750
	3 x 10^4 C3H.OH	7900 ± 1010	55800 ± 2130	37800 ± 3010
	3 x 10^4 F_1	11000 ± 4220	41500 ± 4680	58000 ± 8310
	20% C3H.A	310 ± 70	15940 ± 5690	
	10% C3H.A	220 ± 10	14380 ± 1940	
	20% C3H.OH	870 ± 320	17020 ± 3130	
	10% C3H.OH	180 ± 40	12260 ± 260	
	none	110 ± 20	1090 ± 510	

[1] As described in Table 2, except that 5 x 10^3 T cells primed with TNP-18 in CFA per culture were used.

[2] Preparation of APC, see Table 2. Supernatants were prepared according to Erb and Feldmann, and used at the indicated final concentrations.

[3] Free TNP-18 at a final concentration of 200 ug/ml in culture.

[4] See Table 2, except that TNP-18 used.

Table 6: Repeated nylon wool filtration of lymph node cell preparations does not abolish the effect of APC culture supernatants.

T cells[1]	APC or S/N[2]	Response (cpm ± SE) No Ag	Free Ag[3]	bound Ag[4]
(C3H.OH x C3H.A)F$_1$ unfr.		328 ± 9	62076 ± 10551	
	Con A (4 ug/ml)		35934 ± 4240	
(C3H.OH x C3H.A)F$_1$ NWF	3 x 10^4 C3H.OH	5163 ± 494		84986 ± 12332
x 1	10% C3H.A	192 ± 68	17240 ± 958	
	10% C3H.OH	505 ± 64	30710 ± 854	
	none	91 ± 22	545 ± 91	
	Con A (4 ug/ml)	181 ± 78	4184 ± 238	
(C3H.OH x C3H.A)F$_1$ NWF	3 x 10^4 C3H.OH	1337 ± 205		45064 ± 4861
x 2	10% C3H.A	173 ± 34	11500 ± 3712	
	10% C3H.OH	322 ± 45	32080 ± 4382	
	none	168 ± 66	399 ± 150	
	Con A (4 ug/ml)	156 ± 42	5387 ± 679	

[1-4] The remarks of Table 5 (*vid. sup.*) apply to these experiments.

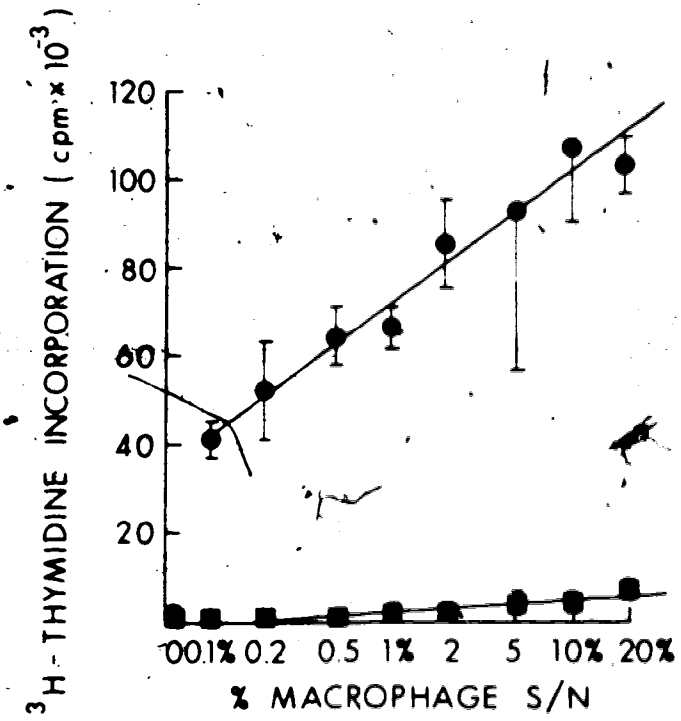


Figure 6: Macrophage culture supernatants stimulate T cell proliferation in the presence of antigen over a wide range of concentrations. 4 x 10^5 CFA-primed purified lymph node cells were cultured with various concentrations of macrophage culture supernatant, in the presence (●) and absence (■) of PPD at 50 ug/ml.

D. Discussion

The H-2 complex has been shown to play an important role in the regul[illegible] cellular interactions in the generation of an immune response. Although initi[illegible] in connection with the control of T-B cell cooperation, the I region of [illegible] implicated in the macrophage – T cell interaction; for example, in the [illegible] of helper T cells (Erb and Feldmann, 1975a) or in the adoptive transfe[illegible]ved h[illegible] hypersensitivity (Vadas *et al*, 1977). All of the preceding is based on [illegible]e of [illegible] techniques, however, and the direct study of this interaction *in vit*[illegible] ad to a [illegible] development of satisfactory T cell proliferation assays. At this junc[illegible] emphasized that although the proliferation assay is measuring mac[illegible] interaction, its *in vivo* correlate is still unclear. Some evidence suc[illegible] proliferating cells are capable of giving rise to DTH on transfer *in* [illegible] yet been determined whether proliferation of helper T cells also contr[illegible] response.

In the present paper, we show that H-2 restriction applies at the level of the macrophage – T cell interaction for both complex (multideterminant) and simple (*Ir* gene controlled) antigens, when macrophage-associated antigen is used. In the case of complex antigens, allogeneic restriction could be clearly seen when possible allogeneic effects were eliminated by the use of tolerant (chimaeric) cells.

Allogeneic restriction at the level of the macrophage – T cell interaction has previously been described in the guinea pig (Rosenthal and Shevach, 1973) and mouse (Vadas *et al*, 1977) for nonchimaeric cells. The persistence of restriction when tolerant cells from one way parent -> F_1 chimaeric mice are used in the proliferation assay apparently contrasts with earlier studies in which it was claimed that the tolerance to alloantigens achieved in a chimaera was sufficient to allow cooperation between chimaeric T cells and B cells of the tolerated haplotype (von Boehmer *et al*, 1975a). These studies were carried out with tetraparental bone marrow chimaeras *A* + *B* -> (*A* x *B*)F_1, repopulated with *A* and *B* lymphoid and reticuloendothelial cells, and which were shown to be able to generate helper cells able to cooperate efficiently with B cells of either genotype. In light of our present knowledge, alternative explanations can be proposed. Several groups have shown independently that the thymic haplotype determines the potential restriction specificities of the lymphocytes processed by it,

while the reticuloendothelial (RE) cells in the periphery specify those which are in fact expressed (Zinkernagel *et al*, 1978a,c; Waldmann *et al*, 1979). Thus, in the two way chimaera, *A* and *B* stem cells are processed through an F_1 thymus and acquire the potential to cooperate with cells of either type. At the peripheral level, these cells encounter reticuloendothelial cells of both haplotypes; hence no haplotype preference is established in priming, and restriction is not seen. It should be noted that our own experiments with two way chimaeras in the proliferation assay do not support this view (*vide infra*).

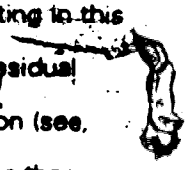
By contrast, in the one way chimaeras, the differentiating stem cells again acquire the potential to cooperate with cells of either haplotype, but show H-2 restriction as a consequence of the presence of RE cells of only one haplotype in the priming environment. It has been shown that these cells are nonetheless capable of recognizing the opposite parental haplotype through the use of mixed chimaeras $A + F_1 \rightarrow F_1$ (Sprent and von Boehmer, 1979), priming of chimaeric cells in a lethally irradiated host of the opposite haplotype (Waldmann *et al*, 1978), and *in vivo* priming of chimaeras with exogeneous APC of the opposite haplotype (Erb *et al*, 1979). Our results are consistent with this model, and confirm its applicability to the macrophage - T cell interaction.

Macrophage level *Ir* gene expression has previously been documented for a number of antigens (Shevach and Rosenthal, 1973; and Yano *et al*, 1978). More recently, a (T,G)-A--L specific *Ir* gene expressed at the macrophage and B cell levels of mice of the H-2*a* haplotype has been described (Marrack and Kappler, 1979); conceivably this might correspond to the situation observed here, also in H-2*a* mice. The antigen used in our studies (TNP-18) differs from most of the polymers used heretofore in having a strictly limited range of recognizable antigeneic determinants; in this respect it is most like the insulin system described recently (Rosenthal, 1978). The relationship between *Ir* gene expression at the macrophage level for the latter antigens, and the random copolymer GAT, which is known to induce suppressor T cells, deserves further comment. The available data suggest that in nonresponders to TNP-18 and presumably to insulin, the principal if not the sole defect lies at the macrophage, which is unable to process antigen and present it in an immunogenic form. This conclusion is based at least in part on the observation that TNP-18 associated with nonresponder macrophages

(C3HA) cannot prime a responder -> F_1 chimaera for an antibody response, while the same macrophages could prime for the response to a multideterminant antigen (Brown, Singh, and Diener, unpublished). This contrasts with the results previously obtained for GAT (Pierce *et al*, 1976), and is consistent with expression of *Ir* genes by the macrophage, particularly in light of the results of Zinkernagel (1978).

It has recently been shown that depletion of macrophages from GAT responder cells permitted the generation of suppressor T cells *in vitro*, and from this result it has been suggested that the induction of suppressor cells has a lower macrophage requirement than the induction of helper cells (Pierres and Germain, 1978). If this result can be confirmed for nonresponder animals, *Ir* gene expression at the macrophage level in animals of suppressor strains is not appreciably different from that in absolute nonresponders, although the presence of suppressor cells would be an additional complication.

In our studies, allogeneic restriction could not be shown for soluble, as opposed to APC-bound, TNP-18 or PPD in culture. The former conflicts with the findings of Rosenwasser and Rosenthal (1978) for the *Ir* gene controlled antigen GLT^{13}; while the latter is consistent with our results. Our results suggest the existence of distinct antigen presenting mechanisms, depending on the physical state and concentration of the antigen. For cell associated antigen *in vitro* and normally *in vivo*, the availability of antigen is limiting, and an *Ia* dependent pathway of T cell activation is followed, perhaps involving direct cell-cell interaction. As recognition is presumably mediated by *Ia* determinants, allogeneic restriction of *Ir* gene expression is seen. A different mechanism may act when high concentrations of soluble antigen are used. Antigen is no longer limiting in this situation, and the primed T cell may be directly triggered by antigen, as residual macrophage function is apparently insufficient to account for presentation (see, however, Mizel and Ben-Zvi, 1980). Macrophage function is limiting under these conditions and our results suggest that nonspecific macrophage factors may augment the proliferative response.



In both of these instances, the mechanism is independent of the structure of the antigen or its degree of complexity, in contrast to the model of Rosenwasser and Rosenthal (1978). Our results are consistent with the concept of a multideterminant

antigen as a collection of simple *Ir* gene controlled determinants, the overall response to the antigen being the sum of the responses to the individual determinants (Okuda *et al*, 1978). By this view, the same presentation mechanism should operate for both simple and complex antigens, and our results suggest this to be the case.

V. MHC restriction of the T cell - antigen presenting cell interaction. II. Two way foetal liver chimaeras and tetraparental mice

A. Introduction

The ways in which the major histocompatibility complex (MHC) regulates the cellular interactions involved in the immune response has been the subject of intense study over the last decade. A number of model systems have been examined, including *in vitro* (Pierce *et al*, 1976) and *in vivo* (Katz, 1977b) T - B cell cooperation, *in vitro* T cell proliferation (Rosenthal, 1978), and the *in vivo* induction of delayed-type hypersensitivity (DTH) (Miller and Vadas, 1977). The *in vitro* T cell proliferation assay has a number of advantages in studies of this sort, chiefly with respect to its simplicity and the relative ease of defining the cooperating cell types, and it has contributed greatly to our appreciation of allogeneic restriction at the level of the T cell - antigen-presenting cell (APC) interaction. The early studies of Rosenthal and Shevach (1973) demonstrated convincingly the requirement for syngenicity between T cells and APC for cooperation, and also the expression of Immune Response (*Ir*) genes by the APC (Shevach and Rosenthal, 1973), results which have been confirmed in subsequent work (Yano *et al*, 1978; Brown *et al*, 1979).

The importance of the thymus in the development of immune competence has long been appreciated, although it is but recently that MHC-controlled functions have been ascribed to it. The work of Zinkernagel led to the hypothesis that the thymic epithelium has a primary role in setting the T cell restriction repertoire of cells differentiating through it, and that the expression of this repertoire can be controlled by the cells of the peripheral reticuloendothelial system during priming (Zinkernagel *et al*, 1978a,b). Studies by a number of groups, including our own, appeared to confirm the validity of this model, and its applicability to both (K, D) and I-region restricted interactions (Sprent and von Boehmer, 1979; Brown *et al*, 1979). Nonetheless, sufficient reports have appeared to suggest limits to this scheme (Matzinger and Mirkwood, 1979; Katz *et al*, 1980b).

A key prediction of the Zinkernagel hypothesis is that under appropriate priming conditions, parental T lymphocytes processed by an F_1 thymus should be capable of

recognizing either of the haplotypes represented in the thymic epithelium as 'self', and thus apparently cooperate in an H-2 unrestricted fashion. In this paper, we use the proliferation assay to directly investigate this point, using both two-way foetal liver chimaeras ($A + B \rightarrow (A \times B)F_1$. Similar considerations should apply to T cells derived from tetraparental mice ($A \leftrightarrow B$), resulting from the fusion of H-2 incompatible eight cell embryos. In preliminary studies, these cells appear to show cooperative preferences reflecting the relative proportions of somatic cells constituting the animals.

B. Materials and Methods

Antigens: Purified Protein Derivative of tuberculin (PPD) was purchased from Connaught Medical Research Laboratories, Willowdale, Ontario.

Media: RPMI-1640-HEPES without added serum was used for incubation of APC with antigen; and RPMI-1640 with bicarbonate buffer (3.5 g $NaHCO_3$ per liter), supplemented with 10% heat-inactivated normal human serum and gentamicin sulphate (50 ug per ml) was used for cell cultures ('culture medium').

Cell preparations: Lymph node cell suspensions were prepared as described in 'General Materials and Methods'. The preparation of peritoneal exudate cells, and the techniques for antigen-pulsing are detailed in Chapter IV.

Chimaeras: (C3H.OH x C3H.A)F_1 mice were lethally-irradiated (950 rad) and reconstituted after 24 hours by the intravenous injection of 10^7 13-day anti-Thy 1.2 + C'-treated foetal liver cells from either or both parental strains. Survival varied between 65 and 90%. One-way chimaeras were > 90% reconstituted with cells of donor origin, and two-way chimaeras showed a balanced chimaerism on testing with appropriate cytotoxic antisera, using a two-stage dye-exclusion technique.

For the production of tetraparental mice, 8 cell embryos were taken from the oviducts of female mice at day 2-1/2 of pregnancy, and the zonae pellucida removed by treatment with pH 2.5 Acid Tyrode's solution. Pairs of embryos were aggregated in drops of Whitten's WK-14 medium under a layer of mineral oil, and placed in culture at 37° C for 24 hours. Mosaic blastocysts were recovered, and transferred surgically into the uteri of female mice on the second day of pseudopregnancy (Wegmann, 1965).

Immunizations: Mice were immunized by injection into both hind footpads of a total of 0.1 ml of an emulsion consisting of equal parts of Complete Freund's Adjuvant (0.5 mg per ml heat killed *M. tuberculosis* H37Ra) (Difco Laboratories, Detroit, MI.) and saline. The popliteal lymph nodes were removed at between 7 and 9 days after priming, and cell suspensions prepared as described above.

Antisera: Alloantisera (A.TH x C3H.OL)F_1 anti-C3H (anti-K*k*) and (A.TL x B10.A)F_1 anti-B10.BR (anti-D*k*) were obtained from Dr. T.L. Delovitch of the University of Toronto. The 50% titres of these sera were 1:2560 and 1:1280, respectively. A monoclonal antibody (11.41) anti-K*k* was obtained from the Salk Institute, San Diego, California, and grown in ascites form in the Department. Dr. T.G. Wegmann kindly provided a hyperimmune C3H.HeJ anti DBA/2J serum (anti-H-2*d*), of titre 1:512.

Treatment with anti-H-2 Sera: See 'General Materials and Methods'. Guinea pig complement was obtained from Flow Laboratories, Inglewood, CA., and agarose absorbed according to the protocol of Cohen and Schlesinger (1970).

Cell culture technique: The T cell proliferation assay of Lee and co-workers (1979) was used. See Chapter IV for details of the protocol. An overall schema for these experiments is given in Figure 6.

C. Results

Experimental Design

The foetal liver chimaeras used as donors of primed T lymphocytes were prepared by reconstituting (C3H.OH x C3H.A)F_1 mice that had been lethally-irradiated (950 rad) 24 hours previously with 10^7 anti-Thy 1.2 + C' treated 13 day foetal liver cells of strains C3H.OH or C3H.A, or 5 x 10^6 cells from each parental strain. Analysis of fully-reconstituted one-way chimaeras indicated that they were >95% reconstituted with lymphoid cells of donor origin, while two-way chimaeras were approximately balanced in their chimaerism. (Range of lymphoid chimaerism, 35:65 to 65:35, C3H.OH:C3H.A). Long-term survival of irradiation chimaeras was > 65%.

Tetraparental mice were derived from Balb/cCr and C3H.HeJ strains; the donor derivation of the somatic tissues, as assessed by coat color ranged between 90:10 and 40:60 (Balb:C3H). Lymphoid chimaerism of individual animals was determined separately

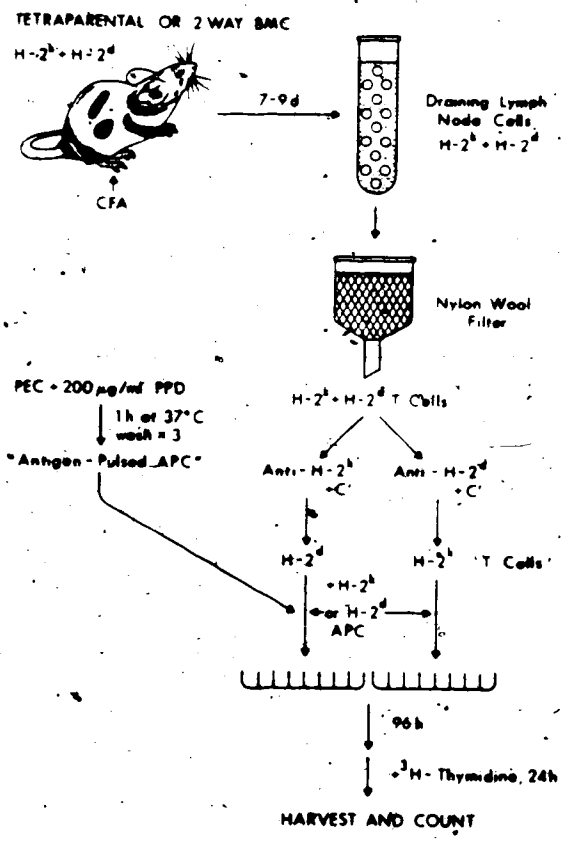


Figure 7: Outline of T cell proliferation assay as modified for two way chimaeric and tetraparental cells. (After Lee *et al*, 1979) T cells of single haplotypes are recovered from the initial chimaeric mixtures by treatment with appropriate alloantisera *in vitro*, and cultured with antigen-pulsed APC as described previously.

for each, and is shown in Table 7.

It was necessary to obtain purified T cells of a single haplotype from the initial chimaeric mixtures, to examine the restriction patterns of primed cells derived from the two-way foetal liver chimaeras and the tetraparental mice. Nylon wool filtered lymph node cell suspensions were divided into two parts, and each was treated with alloantibody and complement, to deplete cells of one haplotype. The remaining purified homozygous T cells, and antigen-pulsed or unpulsed peritoneal APC were placed together in culture for 5 days; proliferation was assessed by incorporation of ^{3}H-thymidine.

H-2 restriction between T cells and APC for primed T cells from one-way and two-way foetal liver chimaeras

Our earlier results with the T cell proliferation assay indicate that parental cells derived from single parent -> F_1 foetal liver chimaeras retain their original restriction pattern, cooperating only with syngeneic APC *in vitro*, after *in vivo* priming (Brown *et al*, 1979). This result excludes the possibility that tolerance to H-2 antigens *per se* is sufficient to allow cooperation across MHC differences, but it fails to address the means by which restriction is established, and the level at which it acts. To approach this point, the H-2 restriction patterns of cells of a single haplotype recovered from *in situ* primed two-way foetal liver chimaeras were studied. Purified T cells of either donor strain gave a proliferative response only when confronted in culture with antigen presented on syngeneic APC (Table 8). Under no circumstances was proliferation above background levels induced by allogeneic, antigen-fed APC. Cells from one-way chimaeras were run as controls, and again gave a proliferative response only with syngeneic APC, confirming our earlier results. Allogeneic restriction of the T cell - APC interaction can thus equally be shown for T cells from two-way and one-way foetal liver chimaeras, under the priming conditions used.

Table. 7: Chimaerism of individual tetraparental mice.

	Somatic		Lymphoid	
Animal	C3H	Balb	C3H	Balb
Chi 1	60	40	68	29
Chi 2	20	80	27	65
Chi 3	10	90	34	60

Table 8: Allogeneic restriction of the T cell - APC interaction, for one-way and two-way fetal liver chimeric cells, in the T cell proliferation assay.

T cells[1]	APC[2]	Response (cpm ± SEM) no Ag[3]	Response (cpm ± SEM) bound Ag[4]
C3H.A -> F_1	C3H.A	488 ± 139	106531 ± 5758*
	C3H.OH	480 ± 332	392 ± 219°
C3H.OH -> F_1	C3H.A	449 ± 204	370 ± 100°
	C3H.OH	125 ± 21	85055 ± 23537*
C3H.A from (C3H.OH + C3H.A) -> F_1	C3H.A	386 ± 117	41194 ± 2930*
	C3H.OH	49 ± 7	245 ± 52+
C3H.OH from (C3H.OH + C3H.A) -> F_1	C3H.A	437 ± 57	355 ± 10°
	C3H.OH	465 ± 273	77315 ± 10444*

[1] T cell donors were primed 8 days previously in both hind footpads with a total of 0.1 ml of an emulsion of equal parts of Complete Freund's Adjuvant and saline. preparation of cell suspensions and culture conditions are described in *Materials and Methods*. 4 x 10^5 cells were used in each culture.

[2] Irradiated (1500 rad) cells obtained from the peritoneal washings of normal mice. Results are shown only for the optimal APC number (3 x 10^4 cells per culture).

[3] Macrophages at 1 x 10^7 cells per ml incubated with PPD (200 ug/ml) for 1 h at 37°, washed x 3 with medium, and diluted to the appropriate final number.

*P < 0.001
+P < 0.05
°P: not significant

T cells derived from tetraparental animals show H-2 restricted interactions with APC in vitro (preliminary studies)

The state of tolerance induced in stem cell reconstituted, irradiation chimaeras depends on a number of factors, including the maturity of the reconstituting inoculum, and its T cell content. Transfer of immature stem cells into an irradiated adult animal results in tolerance in the transferred population, but the mechanism of tolerance induction may be quite different from that obtained during the normal ontogeny of the immune system. Tetraparental mice represent a means by which stable chimaerism, eventually extending to both somatic and lymphoid cells, can be induced long before the emergence of the immune system (McLaren, 1976). They provide an ideal means of examining the H-2 restriction patterns of allogeneic cells differentiating together under physiological conditions.

Three tetraparental chimaeras have been examined. T cells from each animal were assayed separately, in view of the variation in chimaerism encountered. Cells from the best-balanced chimaera (Balb:C3H, 40:60) showed unrestricted cooperation, both Balb and C3H cells cooperating with Balb and C3H APC (Chimaera 1, Table 9). The remaining tetraparentals showed a predominance of Balb haplotype, (Balb:C3H, 80:20 and 90:10). Balb cells from these animals were restricted chiefly to Balb APC. Against expectations, however, C3H T cells from the same animals were also restricted to Balb. The latter two chimaeras showed little if any proliferation with syngeneic (C3H) APC (Chimaeras 2 and 3, Table 9).

These results suggest that restriction patterns are imposed by the somatic tissues of the animals, balanced tetraparentals being functionally unrestricted, and animals showing unbalanced chimaerism, having restriction patterns biased by the predominant H-2 type of the animal.

D. Discussion

The thymus and reticuloendothelial system have been claimed to strongly affect the H-2 restriction patterns of cells differentiating under their influence. The initial observations of Zinkernagel *et al* (1978a,b,c) on the induction of CTL were soon extended to the *in vivo* (Sprent and von Boehmer, 1979; Waldmann *et al*, 1978, 1979)

Table 9: T cells from allophenic mice show variable H-2 restriction patterns in the T cell proliferation assay, reflecting the somatic derivation of the animal.

Animal	T cells[1]	APC[2]	Response (cpm ± SEM) no Ag[3]	Response (cpm ± SEM) bound Ag[4]
normal Balb	Balb	Balb	648 ± 54	26584 ± 4311*
normal C3H	C3H	C3H	5585 ± 1151	30302 ± 2804*
Chi1 (B40:C60)	Balb	Balb	8126 ± 462	38226 ± 1248*
		C3H	4340 ± 916	19370 ± 1550*
	C3H	Balb	5186 ± 695	42317 ± 7005*
		C3H	4720 ± 566	17141 ± 1249*
Chi2 (B80:C20)	Balb	Balb	9029 ± 1853	33379 ± 4136*
		C3H	6727 ± 558	13823 ± 1249+
	C3H	Balb	6688 ± 867	23615 ± 1535*
		C3H	9008 ± 288	13793 ± 189°
Chi3 (B90:C10)	Balb	Balb	6502 ± 1166	25308 ± 1534*
		C3H	7959 ± 235	8359 ± 266°
	C3H	Balb	10154 ± 647	44528 ± 2108*
		C3H	8642 ± 332	8912 ± 546°

[1-4]: The remarks of Table 1 (above) apply.
*P<0.001
+P<0.05
°P: not significant

and *in vitro* (Erb *et al*, 1978, 1979) induction of helper T cells for antibody production, and to the *in vitro* T cell proliferation assay by the group of Schwartz (Longo and Schwartz, 1981; Schwartz *et al*, 1979), and by us (Brown *et al*, 1979). All of these results are compatible with the notion that the thymus sets the potential restriction specificities of cells differentiating under its influence, and that the peripheral reticuloendothelial system subsequently selects out particular specificities for expansion.

Nonetheless, a number of groups have described results at variance with this concept, chiefly in the generation of CTL (Bevan, 1977; Matzinger and Mirkwood, 1978; Doherty and Bennink 1979; Blanden and Andrew, 1979). In this paper, we describe another apparent exception to this principle, this time using the *in vitro* T cell proliferation assay. T cells derived from one and two-way foetal liver chimaeras primed *in situ* were shown to be restricted to cooperation with self-H-2, while preliminary studies with tetraparental mice indicate the possibility of modulation of the T cell cooperating phenotype by the environment.

The work of Swierkosz *et al* (1978) and Sprent (1978a-d) with normal F_1 cells indicates that they comprise two distinct subpopulations, each restricted to cooperation with one parental haplotype. Sprent and von Boehmer (1979), Sprent *et al* (1980), and Waldmann *et al* (1978, 1979) extended these studies to parent -> F_1 stem cell chimaeras, obtaining identical results. The simplest interpretation of this is that the apparent H-2 restriction seen for cells from one-way bone marrow chimaeras is simply a reflection of the restricted APC axis present in priming, and can be overcome by priming in a suitable environment. Erb *et al* (1978) claimed, however, that T cells of a single genotype from *A* + *B* -> (*A* x *B*)F_1 bone marrow chimaeras were restricted to cooperation with syngeneic APC in the primary *in vitro* generation of T helper cells, but that this restriction disappeared after chronic *in vivo* priming.

We wished to extend our earlier findings in the T cell proliferation assay system with one way chimaeras, to verify these predictions.

Our T cell proliferation assay protocol requires that cell donors be primed *in situ*, rather than in adoptive hosts. We therefore took two approaches to allow priming of chimaeric parental cells in the context of syngeneic and allogeneic APC. Two-way foetal liver chimaeras were constructed by repopulating lethally-irradiated adult (C3H.OH x

C3H.A/F$_1$ animals with equal numbers of 13-day C3H.OH and C3H.A anti-Thy 1.2 treated foetal liver cells. The fully reconstituted animals showed a balanced lymphoid chimaerism, with approximately equal representation of both donor haplotypes. We have also studied a limited number of tetraparental mice, derived by *in vitro* fusion of eight cell embryos of C3H.HeJ and Balb/cCr strains. The extent of chimaerism in these animals showed greater variability than in those reconstituted with stem cells, a point of some importance.

Each of these manoeuvers should result in mice, the lymphoid and RE systems of which comprise mixtures of cells of different haplotypes, all mutually tolerant. Thus, it should be possible in theory to prime T cells in the context of syngeneic and allogenic APC. There exist, however, a number of important differences between the two systems. The ontogenic stage at which chimaerism is induced, and thus perhaps the mechanism of tolerance induction, are markedly different, occurring long before the emergence of the lymphoid system in tetraparentals, and in the adult animal in stem cell chimaeras. The derivation of the somatic tissues (including the thymic epithelium) also differs, the tetraparentals' being a mosaic of the parental types, and the chimaeras', F_1 expressing codominantly both parental H-2 types. Additionally, the properties of T cells from stem cell chimaeras may be influenced by residual host-derived radioresistant reticuloendothelial cells (Longo and Schwartz, 1980b).

In situ primed T cells of a single haplotype were recovered from the initially mixed chimaeric cell populations by treatment with appropriate cytotoxic antisera. Based on the considerations outlined above, we expected that homozygous parental T cells derived from the chimaeras should cooperate with APC of either parental haplotype *in vitro*. To our surprise, the two way chimaeric cells were able to proliferate only to antigen associated with syngeneic APC. Rather more complex results were obtained with the tetraparental animals. Cells from the best-balanced chimaera (Chimaera 1) were able to cooperate in an H-2 unrestricted fashion, fulfilling our initial prediction. The other two animals studied (Chimaeras 2 and 3) had a predominance of Balb/cCr haplotype, and lymphoid cells of either Balb/cCr or C3H.HeJ genotypes were restricted to cooperation with Balb APC. This suggests that the environment in which differentiation takes place is crucial in determining the T cell restriction phenotype.

Our finding that two-way chimaeric cells are restricted to self is incompatible with most previous experimental work, save that of Erb *et al* (1978, 1979). These authors showed unprimed T cells from two-way chimaeras to be H-2 restricted in the *in vitro* induction of helper T cells. It may be argued, on this basis, that our brief *in vivo* priming may not be of sufficient duration to overcome a natural haplotype self-preference, and that T cells, if not absolutely restricted, show a preference for syngeneic APC before priming. Longer duration priming, by contrast, may overcome this preference. Such an hypothesis is attractive, as it accounts for our observations, and most of those made by others.

It does not, however, explain the findings of Schwartz *et al* (1979), and of Longo and Schwartz (1981), who claimed two-way chimaeric T cells to be H-2 unrestricted, using a T cell proliferation assay similar to ours. We believe technical factors may be sufficient to explain this discrepancy. In their initial paper, Schwartz *et al* (1979) prepared two-way bone marrow chimaeras according to the protocol of von Boehmer *et al* (1975a,b). Mature T cells were removed from bone marrow by *in vitro* treatment with a commercial anti-theta antiserum, and the adequacy of T cell depletion assessed by the elimination of the Con A proliferative response. Studies by Mosier (1973, 1974) and Mosier and Cohen (1975) have shown that the Con A response appears relatively late in the ontogeny of the immune system, at about 20 days of gestation. Thus, substantial numbers of early postthymic T cells may have been transferred in the stem cell inpculum. By contrast, 13-day foetal liver, as used by us, is taken at about the time of lymphoid development of the thymus (12 - 12-1/2 days; Moore and Owen, 1967a), and prior to peripheral seeding by postthymic cells (Stutman, 1978). It is significant that Longo and Schwartz (1980a) subsequently described substantial modifications to their T cell depletion protocol. Additionally, the Schwartz group used the assay of Corradin *et al* (1977) to assess T cell proliferative responses. In our laboratory, we have found this method to give high backgrounds, and the presence of 2-mercaptoethanol in the culture medium lessens the APC dependence of the response (Lee *et al*, 1979). Shih *et al* (1980) have recently described efficient transfer of antigen to residual APC contaminating cell preparations, and it would seem desirable to maximize the dependence on added APC. In this regard, we have found macrophage culture supernatants, having 2-ME-like activity,

to be able to overcome *Ir* controlled antigen presentation by antigen-pulsed NR APC to (R x NR)F_1 T cells (Chapter IV, and Brown *et al*, 1979).

More recently, Longo and Schwartz (1981) have claimed unrestricted T cell - APC cooperation for T cells derived from tetraparental bone marrow chimaeras. The chimaeras were prepared in this instance with extensively T cell depleted adult bone marrow, the donors being pretreated with ATS and cortisone acetate, and the marrow treated *in vitro* with rabbit anti-mouse brain antiserum. T cell proliferation was assayed, however, in the presence of soluble antigen at the high concentration of 100 ug/ml, rather than with antigen-pulsed APC. We have previously shown that soluble antigen in culture, in the presence of APC is capable of overcoming normal H-2 restricted or *Ir* gene controlled antigen presentation (Brown *et al*, 1979), a possibility which is not excluded in the Schwartz experiments.

Our results with the tetraparental mice are compatible with the work of Erb *et al* (1979), showing unrestricted cooperation for unprimed cells. These authors do not discuss the degree of chimaerism of the animals studied, but if they were approximately balanced, behaviour equivalent to our chimaera 1 might be expected. Chimaeras 2 and 3, however, show restriction although to non-self haplotype in some instances. Even if this simply reflects a predominance of Balb APC in these animals, it argues that, unlike the situation in bone marrow chimaeras, where only self-restriction can be shown in unprimed animals, the cooperating preferences in unprimed tetraparentals can be modulated away from self. This suggests that quite different processes may occur during differentiation in the two situations. We propose that this may be related to the time of induction of chimaerism, and a possible dissociation between the induction of tolerance, and the ability to cooperate across H-2 differences. In the tetraparentals, chimaerism is induced at a stage prior to the setting of self recognition specificities, and also prior to the development of tolerance to 'self'. In stem cell chimaeras, by contrast, some degree of self recognition is established in the donor cells by the time of reconstitution, which can be overcome by appropriate priming.

VI. MHC restriction in the in vivo antibody response. Antigen presenting cell - B cell restriction and the absence of T - B cell restriction

A. Introduction

Analysis of the cellular requirements for a humoral antibody response to thymus-dependent antigens has revealed that three cell types - T cells, B cells, and accessory or antigen-presenting cells (APC) - are necessary. Further studies, with a view to elucidating the regulatory role of the major histocompatibity complex in cell - cell interactions, have yielded variable and frequently confusing results. It was early established that T cells and APC have to share MHC encoded determinants for the *in vitro* proliferative response to antigen (Rosenthal and Shevach, 1973), and that T and B cells have to be syngeneic at the I-A subregion of H-2 for cooperation *in vivo* (Katz *et al*, 1973b). By contrast, homozygous T cells which had undergone differentiation in a heterozygous environment were found to cooperate efficiently with B cells of either parental haplotype *in vivo* (von Boehmer *et al*, 1975a).

These and similar studies led to the concept of adaptive differentiation; that the cooperating phenotype of the T cell may be altered by the environment in which differentiation takes place (Katz, 1977b). A possible mechanism for this adaptive differentiation was suggested in the work of Zinkernagel *et al* (1978a,b,c), whose studies indicate that the T cell recognition repertoire is controlled by both the thymic epithelium and the reticuloendothelial system of the host. Accordingly, genotypic identity alone is not necessarily sufficient to allow cooperation between immunocompetent cells, and it appears that successful interaction requires recognition of MHC determinants expressed on at least some of the cooperating cells (Shih *et al*, 1980; Singer *et al*, 1979).

Recent studies, in which F_1 T cells were selected to antigen in a parental environment suggest that the helper T cell has to recognize determinants expressed simultaneously on both accessory and B cells (Sprent, 1978), while work with cells from one-way chimaeras shows that the chimaeric environment is able to modulate the cooperating preferences of both T and B cells (Katz *et al*, 1978).

Much of the work on allogeneic restriction of cell cooperation has neglected the role of the antigen-presenting cell in the T - B cell cooperative step. In the studies

reported here, we have examined the H-2 requirements for successful interaction between T and B cells in the presence of APC of defined, homozygous genotype. By so doing, we established that the interaction between APC and B cells *in vivo* is under the control of the MHC, and that this restriction is not due to suppressive allogeneic effects.

B. Materials and Methods

Antigens: Keyhole limpet hemocyanin (KLH) was kindly provided by Dr. Marvin Rittenberg, of the University of Oregon Medical School, Portland, OR. Rabbit gamma globulin (RGG) was purchased from the Sigma Chemical Company, St. Louis, MO, and the trinitrophenyl (TNP) derivatives of both antigens prepared by haptenation with trinitrobenzenesulfonic acid (TNBS) (Okuyama and Satake, 1960). TNP-KLH had 8 TNP groups per 100,000 daltons of KLH, and TNP-RGG, 10 TNP per molecule of RGG.

Chimeras: (C3H.OH x C3H.A)F_1 mice were lethally-irradiated (950 rad) and reconstituted within 6 hours by the intravenous injection of 10^7 anti-Thy 1.2 + C' - treated 13 day foetal liver cells from the appropriate parental strain. survival was typically 70-85%, and survivors were >95% reconstituted with cells of donor origin, as assessed by dye-exclusion testing, using cytotoxic alloantisera in a two-stage procedure. Antisera were kindly provided by the Research Resources Branch, NIH, Bethesda, MD, and by Dr. T.L. Delovitch of the University of Toronto.

Immunizations: Mice were immunized by the intraperitoneal injection of 0.1 ml of an emulsion consisting of equal parts of Complete Freund's Adjuvant (0.5 mg/ml heat killed *M. tuberculosis* H37Ra) (Difco Laboratories, Detroit, MI), and a saline solution of KLH or TNP-RGG, to a final antigen concentration of 50 ug/0.1 ml of emulsion. Primed spleens were harvested at 3-6 months after priming, and purified cell suspensions prepared as described. Adoptive secondary recipients were challenged with 0.2 ml of a suspension of alum-precipitated TNP-KLH, containing 2 mg alum and 20 ug TNP-KLH.

Adoptive transfers: Recipient mice were lethally irradiated (850 rad) and given 1 x 10^7 purified T cells intravenously after 24 hours. The following day, 1 x 10^7 B cells were transferred *iv*, followed immediately by alum-precipitated antigen challenge. The secondary response was measured at seven days by estimation of spleen PFC numbers and/or serum antibody levels.

PFC assay: Direct and indirect plaques were determined by the Cunningham - Szenberg slide-chamber technique. (Cunningham and Szenberg, 1968). TNP-sheep red blood cells were prepared according to the protocol of Rittenberg and Pratt (1969).

Serum antibody determinations: Blood was collected from chloroform anesthetized mice by cardiac puncture and allowed to clot. Serum was recovered by centrifugation and stored at -70°C until assayed. Antibody levels were determined by the cellular radioimmunoassay (CRIA) of Longenecker *et al* (1978). Briefly, SRBC were haptenated and washed, and 10^7 cells dispensed into the wells of V-bottomed microtiter trays, containing serial dilutions of the test antisera in saline. After overnight incubation at 4°C, the red cells were washed extensively and further incubated with goat anti-mouse IgG antibody (Cappel Laboratories, Inc., Cochranesville, PA), labelled with ^{125}I. After washing, the cells were resuspended in a small volume of saline, and counted on a Beckmann Bio-Gamma counter for estimation of bound radioactivity. A quaternary hyperimmmune anti-TNP serum was run as a positive control with each experiment, and cpm bound expressed as a fraction of control binding.

C. Results

Experimental design

The chimaeric donors of T and B cells (and adoptive hosts in some instances) were prepared by reconstituting (C3H.OH x C3H.A)F_1 mice that had been lethally irradiated (950 rad) 24 hours previously, with 10^7 anti-Thy 1.2 + C'-treated 13 day parental foetal liver cells (FLC). Analysis of these chimaeras indicated that they were >95% repopulated with lymphoid cells of donor origin, and that the recovered T cells were nonreactive in MLR against the MHC determinants of the tolerated parent. Long term survival of the chimaeras was typically 70-85%, and the animals were free of the signs of graft *versus* host disease. Chimeras were primed with antigen at least three months after reconstitution, and were used as cell donors 3-6 months after priming.

In order to study the role of the APC in the MHC-mediated control of the T - B cell interaction, we transferred KLH primed, chimaeric T cells, and TNP-RGG primed, chimaeric B cells into lethally-irradiated normal or chimaeric hosts, which provided APC function. The hosts were subsequently challenged with alum-precipitated TNP-KLH, and the seven-day antibody response determined (Figure 8). Carrier-primed spleen cell suspensions were passed over nylon wool columns to give a T cell enriched helper population; typically 90% of the nylon wool effluent cells were sensitive to lysis with anti-Thy 1.2 + C'. B cells were purified from hapten-heterologous carrier primed spleen by twice anti-Thy 1.2 + C' treatment, followed by three cycles of adherence to tissue culture grade plastic surfaces. The efficacy of this method of adherent cell depletion has been previously established in our laboratory (Lee *et al*, 1976).

In preliminary experiments, the adoptive transfer system was calibrated by transferring graded numbers of primed syngeneic T and B cells into irradiated syngeneic hosts (Figures 9, 10). Based on these results, T and B cell numbers were chosen such that the response was linearly dependent on the number of each cell type transferred. A clear difference was seen in the helper activity of primed and unprimed T helper cells (Figure

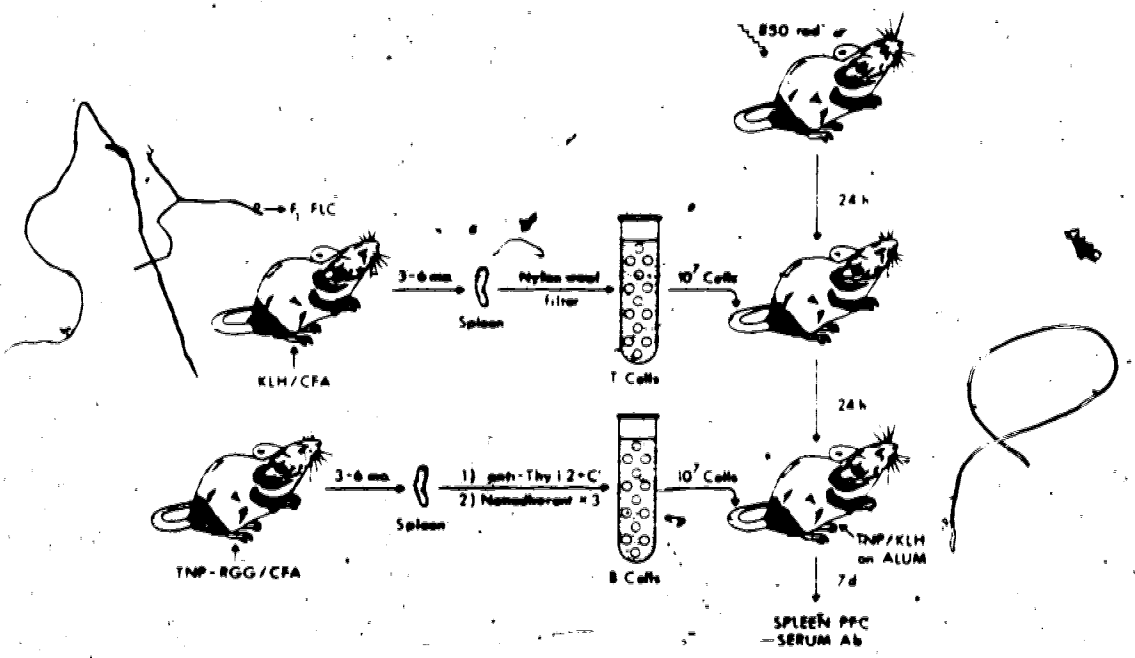


Figure 8: Protocol for adoptive transfer of the antibody response to TNP–KLH. Adoptive recipients of primed T and B cells are lethally irradiated 24 hours prior to T cell transfer. 10^7 purified, KLH primed T cells are given iv., followed 24 hours later by 10^7 purified, TNP–RGG primed B cells iv. The recipients are immediately challenged with 20 ug of alum precipitated TNP–KLH ip., and spleen PFC and serum antibody responses determined at 7 days.

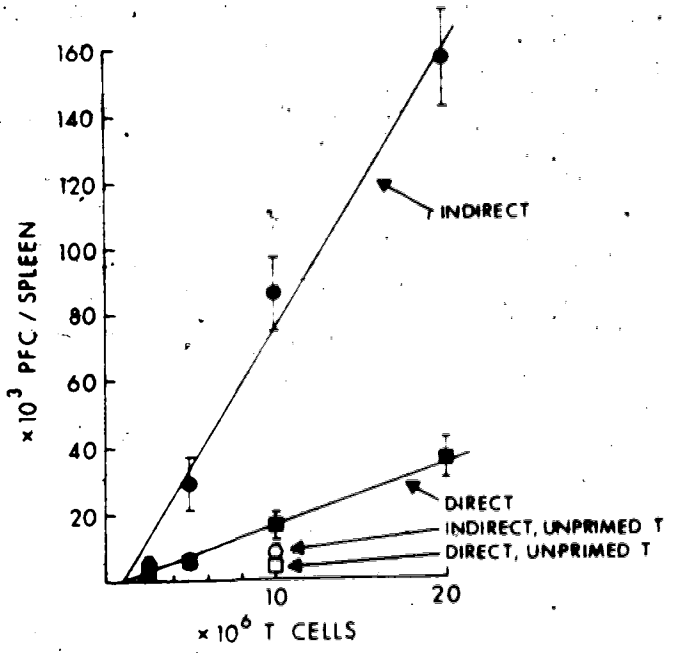


Figure 9: PFC response at seven days as a function of number of T cells transferred. The number of T cells transferred was varied over the range 2.5 – 20 x 10^6, while the B cell number was kept constant at 10 x 10^6

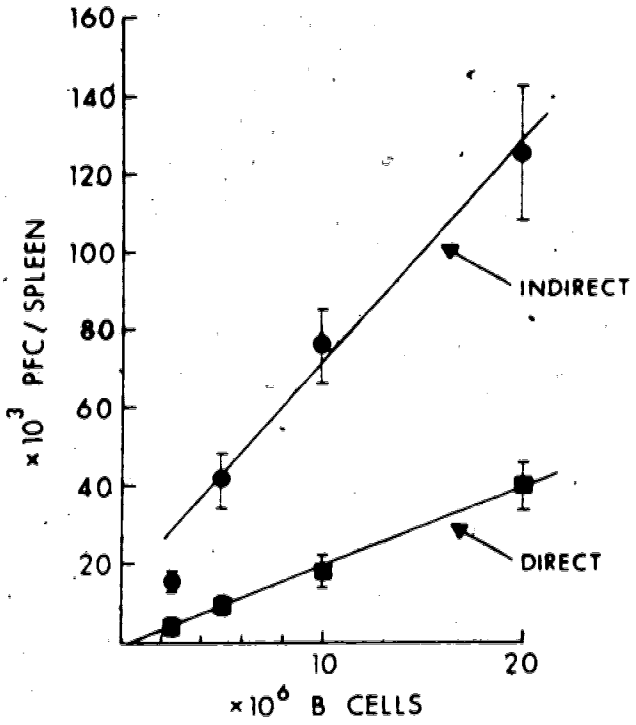


Figure 10: PFC response at seven days as a function of number of B cells transferred. The number of B cells transferred was varied over the range 2.5 – 20 x 10^6, while the T cell number was kept constant at 10 x 10^6.

Cooperation of mutually-tolerant T and B cells across H-2 differences in vivo - role of host haplotype

T and B cells were purified from the spleens of primed chimaeras, removing contaminating cells as described, and transferred into normal parental recipients which had been lethally irradiated 24 hours previously. We were unable to exploit the radioresistance of helper cell function *in situ* described by Katz *et al* (1970a) in view of the potential for allogeneic effects arising from transferring chimaeric cells into fully immunocompetent (and in some instances fully allogeneic) recipients (Hamaoka *et al*, 1973). To further minimize the potential for allogeneic effects between host and transferred cells, the hosts were irradiated 24 hours in advance of cell transfer, a sufficient time to allow depletion of host lymphoid cells.

Histoincompatible but mutually-tolerant T and B cells cooperated efficiently in generating a secondary *in vivo* humoral response against the TNP-hapten, as measured by CRIA, provided that the irradiated host was syngeneic with the transferred B cells (Table 10). Incompatible host - B cell combinations did not show cooperation above background levels, irrespective of the T cell haplotype.

These results thus suggest that the T - B interaction for mutually-tolerant cells is not genetically restricted, while that between the host APC and B cells is under H-2 control. In a repetition of part of the earlier experiments of Katz *et al* (1978), in which H-2 restriction of the T - B cell interaction for chimaeric T and B cells was observed, we transferred primed, chimaeric T and B cells into both 'restricting' (parental) and 'nonrestricting' (F_1) hosts. Our protocol differed from theirs chiefly in the greater purification of the cooperating cell types achieved by us. Based on the above results, we predicted unrestricted T - B cell cooperation in the F_1 host, and this was in fact seen. Parental hosts, by contrast, showed the expected APC - B cell restriction (Table 11, Groups I, II).

Based on these results, it is impossible to exclude that this apparent APC - B cell restriction is not merely an artifact arising from suppression of the normal B cell response by a negative allogeneic effect exerted by the host's residual alloreactive T cells. The following experiments were set up to clarify this point.

Table 10: Host - B cell restriction in the *in vivo* antibody response to TNP-KLH, with chimaeric T and B cells.

Group	T cells[1]	B cells[2]	Host[3]	CPM ± SEM[4]	% bound[5]
I	OH -> F_1	OH -> F_1	OH	7673 ± 275	90 ± 3
II	A -> F_1	A -> F_1	OH	1556 ± 149	18 ± 2
III	A -> F_1	OH -> F_1	OH	7763 ± 1613	91 ± 19
IV	A -> F_1	OH -> F_1	A	1144 ± 282	14 ± 3
V	OH -> F_1	A -> F_1	A	8133 ± 1082	96 ± 13
VI	OH -> F_1	A -> F_1	OH	1850 ± 221	22 ± 3

[1] T cell donors were primed approx. 3 months previously with 50 ug KLH/CFA ip. 10^7 nylon wool filtered spleen cells were given iv to the irradiated host.

[2] B cell donors were primed approx. 3 months previously with 50 ug TNP-HGG/CFA ip. 10^7 anti-Thy 1.2 + C, treated, plastic nonadherent cells were transferred into the irradiated host.

[3] Host animals were irradiated with 850 rad 24 hours before cell transfer. Immediately after cell transfer, the host was challenged with 20 ug TNP-KLH/alum ip. Blood for determination of serum antibody levels taken at 7 d.

[4] 7 day serum antibody levels determined by cellular radioimmunoassay. cpm represents ^{125}I goat anti-mouse IgG bound to the washed TNP-SRBC pellet.

[5] Relative to hyperimmune IgG anti-TNP serum.

Significance levels: Group I *vs* Group III, n.s.; Group I *vs* Group IV, $P < 0.001$; Group V *vs* Groups II, VI, $P < 0.001$.

Table 11: Lack of restriction for the T - B cell interaction for purified T cells and B cells in the heterozygous host

Group	T cells[1]	B cells[2]	Host[3]	cpm ± SEM[4]	%bound[5]
I	A -> F_1	OH -> F_1	A	946 ± 178	22 ± 4
II	OH -> F_1	OH -> F_1	OH	3571 ± 153	83 ± 4
III	A -> F_1	OH -> F_1	F_1	2624 ± 120	61 ± 3
IV	OH -> F_1	OH -> F_1	F_1	2918 ± 415	68 ± 10

[1-5] See Table 10.

Significance levels: Group II *vs* Group IV, n.s.; Group I *vs* Group III, $P < 0.001$

Negative allogeneic effects are not responsible for apparent H-2 restriction of the host - B cell interaction

Allogeneic effects *in vivo* could potentially be mediated by residual alloreactive host cells recognizing and responding to MHC determinants present on either the transferred T or B cells, and could result in suppression of the B cell response directed against the hapten.

A comparison was accordingly made of the ability of normal and specifically-tolerant hosts to sustain a response. Parent -> F_1 foetal liver chimaeras were used as tolerant, irradiated adoptive recipients; these animals were >95% repopulated with lymphoid cells of donor origin, and were functionally fully reconstituted with donor-derived APC (Sprent, 1980).

To rule out allogeneic effects between host and B cells, parallel adoptive transfer experiments were carried out in which the same T and B cell combinations were transferred into previously-irradiated parental and chimaeric hosts (Table 12).

Strictly comparable results were obtained in both instances, with allogeneic host - B cell pairings failings to cooperate. The levels of serum antibody, as determined by CRIA, closely parallel the seven day spleen plaque forming cell response, and the two measures will therefore be used interchangeably. Both IgM and IgG responses were depressed to an equivalent extent in incompatible host - B cell combinations.

These experiments do not, however, exclude suppression of the B cell response by reaction between the host and transferred T cells. Syngeneic host - B cell combinations with either normal or chimaeric hosts were set up, and the influence of helper T cell genotype examined. Normal and chimaeric hosts provided equivalent cooperating environments, irrespective of the host - T cell compatibility (Table 13).

D. Discussion

Several levels of control of cell - cell interactions by the MHC have been described by various investigators. In this paper, we report on the role of the APC in the regulation of the T - B cell interaction *in vivo*, using an adoptive transfer system in which APC function is provided by the adoptive host. Our results are most compatible with H-2 restriction expressed between APC and B cell, although the possibility of T

Table 12 Negative allogeneic effects between radioresistant host cells and B cells do not account for host - B cell restriction: comparison of normal and chimaeric hosts.

Group	T cells[1]	B cells[2]	Host[3]	PFC/spleen[4] Direct	PFC/spleen[4] Indirect	Antibody % bound[5]
I	A -> F_1	A -> F_1	A -> F_1	38500 ± 2010	170000 ± 10510	49 ± 12
II	OH -> F_1	OH -> F_1	OH -> F_1	41500 ± 3130	138000 ± 6600	46 ± 9
III	A -> F_1	OH -> F_1	OH -> F_1	43500 ± 1120	131500 ± 5140	36 ± 4
IV	A -> F_1	OH -> F_1	A	2700 ± 80	3700 ± 540	16 ± 3
V	A -> F_1	OH -> F_1	A -> F_1	4000 ± 980	3000 ± 450	7 ± 3
VI	OH -> F_1	A -> F_1	OH	2800 ± 630	3400 ± 1430	5 ± 3
VII	OH -> F_1	A -> F_1	OH -> F_1	3600 ± 630	4900 ± 1210	15 ± 4
VIII	OH -> F_1	OH -> F_1	A -> F_1	1200 ± 270	2700 ± 540	9 ± 3
IX	A -> F_1	A -> F_1	OH -> F_1	1500 ± 270	2300 ± 890	1 ± 1

[1][2][3] See Table 10

[4] 7 day splenic PFC response. Assayed on TNP-SRBC.

Significance levels: Group I *vs* Group VI, PFC $P < 0.001$, Ab $P < 0.001$; Group I *vs* Group VII, PFC $P < 0.001$, Ab $P < 0.025$; Group I *vs* Group IX, PFC $P < 0.001$, Ab $P < 0.025$; Group II *vs* Group III, PFC P n.s., Ab P n.s.; Group II *vs* Group IV, PFC $P < 0.001$, Ab $P < 0.05$; Group II *vs* Group V, PFC $P < 0.001$, Ab $P < 0.025$; Group II *vs* Group VIII, PFC $P < 0.001$, Ab $P < 0.001$

Table 13: Alloreactivity between the host and transferred chimaeric T cells is not responsible for apparent host - B cell restriction: equivalence of normal and chimaeric hosts.

Group	T cells[1]	B cells[2]	Host[3]	PFC/spleen[4] Direct	Indirect
I	OH -> F_1	OH -> F_1	OH -> F_1	45000 ± 3500	159500 ± 11000
II	A -> F_1	A -> F_1	A -> F_1	40500 ± 6500	136500 ± 5750
III	OH -> F_1	A -> F_1	A	35000 ± 3500	172500 ± 23500
IV	OH -> F_1	A -> F_1	A -> F_1	37000 ± 5500	180500 ± 11750
V	A -> F_1	OH -> F_1	OH	38500 ± 2500	160500 ± 10000
VI	A -> F_1	OH -> F_1	OH -> F_1	46000 ± 1750	158000 ± 3750

[1234] See Tables 10, 11

Significance levels: Group I *vs* Group V, n.s.; Group I *vs* Group VI, n.s.; Group II *vs* Group III, n.s.; Group II *vs* Group IV, $p = 0.05$

cell - APC restriction in priming, and T - B cell restriction of cooperation cannot be excluded. We were surprised to be unable to show at least H-2 restriction of the T cell - APC interaction, which has been described by Zinkernagel *et al* (1978c) and Waldmann *et al* (1978) in *in vivo* CTL and T - B cell cooperative systems, respectively, and by us for acutely-primed T cells, in the T cell proliferation assay (Brown *et al*, 1979). Possible reasons for this discrepancy are discussed below. The above notwithstanding, it is only under these particular conditions of H-2 unrestricted T cell activity, that APC - B cell restriction can be shown. If, as suggested by some workers, T cells must recognize the same determinants on APC and B cells, T cell - APC restriction will necessarily require concomitant T - B cell restriction, obscuring that between B cells and APC.

It is significant that previous demonstrations of APC - B cell restriction have relied on thymus-independent antigens. Thus, Gorczynski *et al* (1980) used an *in vitro* culture system in which suboptimal concentrations of LPS substitute for T cell help, and Hodes *et al* (1981) used the thymus independent Type 2 antigen, TNP-Ficoll, to show this with an *in vitro* culture system.

The alternative model of T cell - APC and consequent T - B cell restriction proposed by Sprent (1978c,d) and others (Yamashita and Shevach, 1978; Swierkosz *et al*, 1978) is based on the finding that normal F_1 and parent -> F_1 chimaeric T cells contain separate subpopulations of T cells, each restricted to cooperation with one parental haplotype (Swierkosz *et al*, 1978). Under ordinary conditions of *in vivo* priming with F_1 APC or in F_1 adoptive recipients, these populations give the overall impression of unrestricted cooperation, but they can be resolved by adoptive priming in irradiated parental recipients (Sprent, 1978c; Sprent and von Boehmer, 1979).

Our findings of apparent APC - B cell restriction, and the lack of T - B cell restriction could reflect positive or negative allogeneic effects between the irradiated hosts, and the transferred T or B cells. Initially, mutually-tolerant T and B cells were transferred into supralethally irradiated parental hosts. Cooperation was observed only when the APC and B cells were syngeneic, and the response was not influenced by the T cell genotype. This result clearly excludes a negative allogeneic effect between alloreactive radioresistant T cells remaining in the host, and the transferred T cells; a possibility which would have resulted in apparent T - B cell restriction, but it does not

address the possibility of a positive allogeneic effect. The latter would be expected to give a result indistinguishable from the preceding. Additionally, it could be argued that the radioresistant T cells were able to directly suppress the response of B cells not syngeneic to the host. This possibility was ruled out in the experiments shown in Tables 12 and 13, in which irradiated parent -> F_1 chimaeric animals were used as adoptive recipients. These animals were essentially fully repopulated with cells of donor parental origin, and cells recovered from the reconstituted animals were shown to be nonreactive in MLR against the H-2 determinants of the other respective parent. Comparable patterns of H-2 restriction were obtained with parental and chimaeric hosts, suggesting that the role of residual alloreactive cells was insignificant.

Our results can be interpreted in terms of T - MΦ and T - B restriction only if it is postulated that our chimaeric T cells are not restricted to cooperation with a given H-2 prior to transfer into the irradiated recipient. Two possibilities may exist for this; that the T cells are effectively unprimed, or that they have been primed in the context of nonrestricting (F_1) APC *in situ*, and undergo selection to H-2 only in the final host. The first possibility - a lack of priming - can be excluded on the basis of the results in Table 12, Groups X and XI, showing a striking difference in helper activity of primed and unprimed T cells in the adoptive transfer system. Additionally, the character of the response obtained was that of a secondary immune response, with a predominant IgG component. It is impossible to exclude the second possibility definitively, although all our evidence is against it. Our earlier studies in the T cell proliferation assay show clearly that parent -> F_1 chimaeric T cells primed *in situ* are restricted to cooperation with syngeneic APC, in the absence of detectable allogeneic effects. This argues against any substantial component of endogeneous F_1-derived APC in the chimaeras. Given also the time allowed for repopulation of the chimaeras before priming, and the supralethal doses of irradiation used to prepare them, it seems most unlikely that there should be a residuum of F_1 APC, especially in light of Sprent's results, showing disappearance of host-derived APC by 15 weeks (Sprent, 1980). Nonetheless, we cannot exclude that a small number of F_1 APC are present, and that over the three to six month priming period used by us, these host-derived APC may be sufficient to expand both parentally-restricted subpopulations to detectable levels. On purely statistical grounds,

however, the extent of F_1 repopulation would have to be substantial to account for our data.

Additionally, if the unrestricted behaviour of T cells derived from chronic *in situ* priming of one-way FLC is due to significant contamination of the chimaeras by F_1 APC, the equivalence of normal and chimaeric secondary adoptive hosts would not be expected. These chimaeras would presumably contain equivalent proportions of F_1 APC to the priming hosts, and re-selection to antigen would be mediated by both P and F_1 APC; thus B cell - APC restriction would not be seen. Normal hosts, having only parental APC, would show the expected restriction.

Alternative, and to us preferable, explanations include the possibility that prolonged (3 month) and vigorous (antigen in CFA) *in vivo* priming is sufficient to expand up both parentally-restricted T cell subpopulations in the parent -> F_1 chimaeras, irrespective of the APC haplotype repopulating the host. Matzinger and Mirkwood (1978) have obtained equivalent findings in the induction of CTL restricted to minor H antigens associated with non-self H-2, in fully allogeneic stem cell chimaeras, which they attributed to the heroic measures used for priming. Conceivably, chronic *in situ* priming may also induce a single unrestricted T cell population, capable of cooperation with both haplotypes represented in the priming host. Alternatively, the physical form of the antigen present during either T cell induction or cooperation may be significant, as implied in our observation that soluble antigen is capable of overcoming apparent H-2 restriction for primed (and restricted) T cells *in vitro* (Brown *et al*, 1979). Finally, it cannot be excluded that the helper T cells induced by chronic *in vivo* priming do not require restimulation by antigen, at least in association with APC syngeneic to those present during induction.

An interesting point arising from the data in Table 12 is that neither direct nor indirect PFC responses were observed in allogeneic host - B cell combinations. If one takes the view that B cells can undergo adaptive differentiation (Katz, 1980), histoincompatible APC and B cells from parent -> F_1 chimaeras should be able to give an IgM response, the B cells in this case being essentially unprimed to hapten in the context of allogeneic APC, but still able to cooperate. Our finding argues against B cell adaptive differentiation, and is consistent with the work of Sprent and Bruce (1979). The mouse strains used in these studies differ chiefly at the left hand end of H-2, and although our

evidence does not bear directly on this point, it seems likely that the APC - B cell interaction is under I-region control.

A key feature of our experiments, and an aspect largely neglected by previous workers with *in vivo* systems, is the careful depletion of extraneous cell types from the transferred cooperating populations. This is particularly important as it allowed us to directly examine the *in vivo* T cell - APC - B cell interaction. Our earlier work with the T cell proliferation assay established the necessity for only a very few APC in an *in vitro* response, and we thus felt it necessary to rigorously deplete APC from the T and B cells prior to adoptive transfer, to isolate the host's role in the cooperative event. The studies of Shih *et al* (1980), clearly documenting the efficiency of antigen transfer to residual APC in the B cell preparations, emphasize the importance of this step.

Our failure to demonstrate T - B cell allogeneic restriction is at variance with earlier data of Katz *et al* (1978), who initially reported absolute restriction of the T - B cell interaction *in vivo*, using T and B cells derived from one-way bone marrow chimaeras in an adoptive transfer system. Our attempt to repeat a part of these experiments was unsuccessful, as we were unable to show T - B cell restriction in the F_1, as opposed to the parental, host. We believe that this discrepancy simply reflects the H-2 unrestricted help in our assay system, which may in turn be related to the manner of preparation of the chimaeras (foetal liver *versus* bone marrow). Alternatively, the degree of depletion of extraneous cell types from the transferred populations may be significant (*vide supra*). Katz has, however, recently reported that the H-2 restriction of T cells from P -> F_1 chimaeras is considerably less tight than was previously thought, and that the 'pseudorestriction' seen for cells primed *in situ* in the chimaeric environment can be overcome by adoptive priming in the context of appropriate APC. This and other results (Katz, 1979; Katz *et al*, 1979, 1980a,b) have suggested that the original interpretation of the data suggesting adaptive differentiation was overly stringent, and that the true situation may be more one of 'environmental restraint', in which extrathymic influences during differentiation may be of critical importance.

Sprent, using *in vivo* positive selection to antigen of F_1 cells in a parental environment, found that H-2 restriction was imposed during the selection process, a

result not compatible with ours (Sprent, 1978). The same authors, using cells from stem cell chimaeras, have reported unrestricted T - B cell cooperation. T and B cells derived from two-way bone marrow chimaeras were used in an adoptive transfer protocol similar to ours, although with an F_1 host and thus F_1 APC in the ultimate cooperation step. The interaction between T and B cells under these conditions was not restricted by the H-2 genotype, but did reflect the phenotype of the cells, a result which they interpreted in terms of *in situ* priming of the T cell subsets by both APC types (von Boehmer *et al*, 1975). In a subsequent study, parental cells derived from P + F_1 -> F_1 chimaeras were shown to comprise two functionally distinct subpopulations, with cooperative preferences dictated by the H-2 type of the APC with which priming occurred. *In situ* priming here is with both P and F_1 cells, the latter in sufficient proportions to give the overall picture of unrestricted cooperation (Sprent and von Boehmer, 1979). Both of these results can be interpreted in terms of APC - T cell, and T - B cell restriction, as is done by Sprent; they are, however, equally compatible with a model of unrestricted T - B cell cooperation. APC - B cell restriction is not seen in this situation, however, as the hosts for the adoptive transfers are themselves nonrestricting F_1's.

Shih *et al* (1980) and Singer *et al* (1979) have both demonstrated a lack of T - B cell restriction *in vivo* and *in vitro*, and further have claimed to find T cell - APC restriction. It is difficult to reconcile this last observation with our findings, other than on the basis of differences in experimental conditions. It could be argued that their system tends to reveal manifestations of T cell - APC restriction ; for example an H-2 restricted stimulation or restimulation of helper T cells, while our protocol isolates a similarly MHC-restricted cooperative event between APC and B cells. Such a possibility is also suggested by the work of Howie and Feldmann (1978), and Boswell *et al* (1980a,b,c), the latter showing an APC requirement for the activation of the Lyb 5+ B cell subpopulation by both TD and TI antigens.

We are thus left to reconcile our findings with those in the existing literature. It is unlikely that an overall synthesis can be achieved; nonetheless, certain unifying points emerge.

Our data are compatible in broad outline with either the prevailing hypothesis of sequential APC - T cell, and T - B cell restriction, or with our model of APC - B cell

restriction. T cell – APC restriction may be present, but was not revealed under our particular experimental conditions, as was discussed above. At present, it is impossible to decide between these two alternatives definitively, although our evidence is, we feel, more consistent with APC – B cell restriction, and the absence of T – B cell restriction.

Our results thus support the notion that the T cell, once induced, can provide antigen-specific but H-2 unrestricted help, to a B cell population, although this help requires the mediation of APC syngeneic to the B cell to be efficiently delivered; the T and B cells must be mutually-tolerant for this. Others have shown a requirement for APC in the *in vitro* B cell response to TD antigens (Mosier and Coopleson, 1978), and APC – B cell restriction has been demonstrated for the TI antigen response (Hodes *et al*, 1981). Although these results can be interpreted in terms of the alternative hypothesis of H-2 restriction of T cell – APC, and T – B cell interactions, this analysis requires certain assumptions not supported by our data.

VII. General Discussion and Conclusions

This thesis decribes the development and use of two model systems to explore some aspects of H-2 restriction of cooperation of immunocompetent cells.

The greatest potential complication in analyses of this sort is alloreactivity between the cooperating cell types. If not recognized and adequately controlled for, spurious results reflecting nonspecific enhancing or suppressive effects may be obtained.

Historically, a number of approaches have been taken to minimize these so-called allogeneic effects, most involving the induction of specific immunological unreactivity to the H-2 antigens of the cooperating partner cells. Both acute and chronic procedures have been used for this, the former including *in situ* irradiation of primed helper cells to minimize the potential for stimulation by alloantigens (Katz *et al*, 1970a); *in vivo* negative selection to H-2 in a semiallogeneic host (Ford and Atkins, 1971), and deletion of cells responding to alloantigens *in vitro* by exposure to BUdR and light. All of these techniques are relatively inconvenient, and provide no assurance that all alloreactive cells have been eliminated.

Chronic tolerance induction has been used in the great majority of recent studies on H-2 restriction. Kindred (1975) initially described the cooperative preferences of cells rendered neonatally tolerant by the classical Billingham procedure, and von Boehmer *et al* (1975a) were responsible for popularizing the stem cell/irradiation chimaera. Originally, the latter animals were viewed simply as a means of tolerizing cells to foreign H-2 antigens (Sprent and von Boehmer, 1975a). It has subsequently become clear that the behaviour of cells from these animals is rather more complex than would be expected on this basis, and they have proven particularly useful in studies on the cooperative preferences of cells which have undergone lymphoid differentiation in a foreign environment.

In our studies, lethally-irradiated heterozygous (F_1) animals were reconstituted with stem cells of one or both parents. A number of factors affect the 'quality' of the chimaeras obtained, including the use of adequate doses of irradiation, the stem cell source, and the adequacy of depletion of relatively mature cells from the reconstituting inoculum (Longo and Schwartz, 1980a). Our stem cell chimaeras were initially constructed

with 15 day foetal liver cells, a regimen subsequently changed to anti-Thy 1.2 treated 13 day FLC, as the importance of complete elimination of postthymic cells was better appreciated.

T cells from one way [*A* or *B* -> (*A* x *B*)F_1] and homozygous *A* or *B* T cells purified with alloantisera from two way [*A* + *B* -> (*A* x *B*)F_1] FLC primed *in situ* behave identically in our hands, showing apparent clear-cut restriction to self H-2 in the macrophage dependent T cell proliferation assay. Nontolerant T cells gave comparable results in at least some strain combinations, but in others, MLR-like effects masked any antigen specific proliferation (Chapters IV, V; and Brown *et al*, 1979).

The H-2 restricted behaviour of cells from one-way, but not two-way FLC is to be expected in view of the work indicating the dual influence of the thymus and reticuloendothelial system on the T cell repertoire. Our results with two-way chimaeric cells are incompatible, however, with both theory and certain experimental results obtained with a T cell proliferation assay by Schwartz *et al* (1978) and Longo and Schwartz (1981). Erb *et al* (1978, 1979) have obtained results equivalent to ours with cells from two-way BMC, in the APC dependent generation of T helper cells *in vitro*.

We feel that technical differences, as discussed in Chapter V, are sufficient to account for the discrepancy between our findings, and those of Schwartz and his colleagues. We therefore conclude that acutely-primed T cells derived from two-way FLC show at least a 'self preference' in our assay system. Our studies thus far do not allow us to determine if this is in fact an absolute H-2 restriction, but we do not favour this interpretation, particularly in light of Katz' recent work on 'environmental restraint' (Katz *et al*, 1979, 1980a; Katz, 1980).

More recently, we have studied a limited number of tetraparental mice *A* <-> *B*, derived by *in vitro* fusion of H-2 incompatible eight cell stage embryos (McLaren *et al*, 1976) The very early induction of chimaerism, and the relatively normal ontogeny of the immune system in these animals provides, we feel, a more physiological model for the analysis of H-2 restriction patterns than is the case with bone marrow chimaeras. Our work indicates this viewpoint may be correct, as our preliminary evidence indicates that the cooperative preferences of T cells from these animals can be modulated away from [redacted]. In chimaeras having approximately equal representation of both somatic haplotypes,

apparently unrestricted T cell – APC cooperation is observed; while in 'unbalanced' animals, restriction to the predominant somatic haplotype is seen. These results are quite different from ours with the stem cell chimaeras, and are again compatible with those of Erb *et al* (1979), using tetraparental cells in the *in vitro* induction of helper T cells. A number of factors may account for the differences between stem cell and embryo fusion chimaeras. Perhaps the most important is that lymphoid differentiation from stem cells in an irradiated semiallogeneic adult host is quite different from that obtained under physiological conditions of ontogeny. The influence of long-lived host derived cells on the developing immune system in stem cell chimaeras (Longo and Schwartz, 1980) cannot be discounted. The other major point concerns the nature of the stem cells, and the extent to which they have been influenced by the donor environment prior to their being harvested for reconstitution. The importance of thorough depletion of mature T cells from the donor marrow cells to prevnt GvH disease has long been appreciated, although it is only comparatively recently that the effects of immature postthymic T cell precursors (PTP) have been recognized (Stutman *et al*, 1978). We feel that FLC offer substantial advantages over bone marrow in this respect, as it is possible to obtain stem cell populations in good yield shortly after the onset of lymphoid development in the thymus, beginning at 13 days of gestation. Nonetheless, even though these cells should be essentially prethymic, they are less satisfactory than the fusion chimaeras.

Our results, comparing FLC with tetraparentals, suggest that the former have an inherent self preference, which is not completely overcome by influences of the chimaeric host environment during ontogeny, at least in the unprimed or acutely primed state. Chronic or vigourous *in situ* priming, as shown in the work of Matzinger and Mirkwood (1978) and of Erb *et al* (1978, 1979) can, however, overcome this restriction. By contrast, tetraparentals show patterns of cooperation chiefly influenced by the host genotype.

The second major part of our studies examined H-2 restriction of T cell – B cell – APC cooperation *in vivo*. For reasons discussed above, we used T and B cells derived from FLC primed *in situ*, to minimize the potential for alloreactivity between the cooperating cell types. In addition, we rigourously depleted APC from the T and B cells, so that the only functional APC were derived from the adoptive transfer host. By

the use of both of these approaches, it was possible to use homozygous animals (or parent -> F_1 chimaeras) as adoptive recipients, without the complication of alloreactivity, and thus to delineate the role of the APC in cooperation.

We described H-2 restriction between the APC and B cell, without a corresponding T cell - APC, or T - B cell restriction. H-2 restriction between B cell and APC has been previously described, but only in the *in vitro* response to TI-2 antigens (Hodes *et al*, 1981), or in the *in vitro* response to certain TD antigens, in which helper T cell function is replaced by suboptimal amounts of the TI-1 antigen, LPS (Gorczynski *et al*, 1980). The recent work of Boswell *et al* (1980a-c), has made it clear that TI-2 and TD antigens both activate the same Lyb 5+ B cell subpopulation, via an APC dependent pathway. It does not surprise us, therefore, that similar genetic constraints should apply in the responses to the two classes of antigens.

The apparent lack of genetic restriction between T cells and APC is rather puzzling to us, in light of the near universality of this finding by others. We believe that this T cell - APC restriction represents a need for restimulation of helper T cells by antigen, in the particular experimental systems used. Most studies of this sort have relied on short term adoptive priming in irradiated recipients; as discussed above, this may be insufficient to overcome 'self preference' in the T cell population, which thus appears to be H-2 restricted. Long term priming with adjuvants may, by contrast, allow theclonal expansion and expression of an unrestricted T cell cooperative phenotype; or it may circumvent the requirement for H-2 restricted restimulation of T helper cells by APC associated antigen in the adoptive recipient. Persistence of antigen in the primed donor may also be involved in this apparent lack of restriction.

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Appendix I. Is acquired immunological tolerance genetically transmissible?

A. Introduction

The recent work of Gorczynski and Steele (1980, 1981) has indicated that acquired tolerance to major histocompatibility antigens can be vertically transmitted through the male germ line for at least two generations, a finding which appears to violate Weisman's doctrine of the separation of soma from germ-line. As a possible mechanism for this phenomenon, Gorczynski and Steele propose a variant of Temin's hypothesis concerning the possible role of C-type RNA viruses in the transduction of genetic material (Temin, 1971, 1976).

In view of the great importance of such a finding, if verified, we have attempted to reproduce this phenomenon, athough with a somewhat different system. In our protocol, the tolerant male was a C3H/HeJ <-> Balb/cCr tetraparental mouse and was mated to normal C3H/HeJ females. The homozygous C3H/HeJ progeny were subsequently tested for tolerance of H-2*d* MHC antigens by their ability to reject Balb/cCr foetal heart grafts and by the kinetics of clearance of ^{131}IUdR-labelled L1210 leukaemia cells. We find that the first-generation progeny are indistinguishable from normal age-matched homozygotes in their first-set rejection of cardiac allografts, and in their ability to be primed for immune clearance of tumour bearing the relevant antigen *in vivo*. Similar results were obtained in limited studies on heart graft rejection by the progeny derived from mating separated parabiont males with normal females. Thus, these results place strict limits on the general applicability of Steele's theory.

B. Materials and Methods

Mice: C3H/HeJ, DBA/2J, and Balb/cCr mice were obtained from the Small Animal Breeding Program, The University of Alberta, Edmonton, Canada. Plugged female mice of strains C3H/HeJ, and Balb/cCr, and pseudopregnant females of strain ICR were obtained from the same source.

Tetraparental chimeras: Tetraparental mouse chimeras were made according to standard procedures (Wegmann, 1970; McLaren, 1976). Briefly, eight-cell stage embryos of strains C3H/HeJ and Balb/cCr were obtained by flushing the oviducts and proximal

uterine segments of female mice on the second day of pregnancy (day of plugging = day 0). The zonae pellucida of the recovered embryos were removed by brief exposure to pH 2.5 Acid Tyrode's solution, and pairs of embryos were aggregated in drops of Whitten's WK-14 medium under a layer of mineral oil. After *in vitro* culture for 24-36 hours, mosaic blastocysts were transferred surgically to ICR foster-mothers on the second day of pseudopregnancy. On day 19 of gestation, the mothers were killed, and the young were delivered by Caesarian section. Cannibalism was sharply reduced by fostering the litters onto lactating ICR females.

Parabiont mice: Adult male mice of strains DBA/2J and (C3H/HeJ x DBA/2J)F_1 were surgically parabiosed by suturing the wound margins of opposed longitudinal incisions in the skin of the lateral thoracic and abdominal walls (Shaw *et al*, 1974). After approximately three months of parabiosis, the animals were surgically separated under penthrane anaesthesia, and the skin wounds closed by wound clips.

Fetal heart grafting: Seventeen day Balb/cCr or C3H/HeJ foetal hearts were implanted subcutaneously in pockets at the base of the external ears of the recipient mice (Jirsch *et al*, 1974). Graft acceptance was assessed at various times in anaesthetized animals by electrocardiography, using difference electrodes inserted through the pinnae. This technique, developed by Dr. K.G. Pearson of the Department of Physiology, The University of Alberta, allows recording of the electrical activity of the graft, without interference from the host heart. Spontaneous electrical activity could be detected in grafts by 7-10 days; in case of rejection, no impulses could be recorded after 14-17 days.

Tumor clearance in vivo: The mouse leukaemia L1210 was passaged *in vivo* in Balb/cCr mice. Three days prior to labelling, a final *in vivo* passage was carried out by injecting 10^7 cells *ip* into Balb/cCr recipients. Fifty to 100 microcuries of ^{131}IUdR in Hank's BSS at pH 7.0 to 7.3 was injected *ip* into each tumour bearing mouse, and 4-6 hours later, the labelled cells were recovered, and washed extensively to remove unincorporated label. 10^6 cells, containing approximately 10^3 cpm of label, were injected into each of the mice, which had in all instances been heart-grafted 21 days previously. Clearance of tumour was quantitated by daily whole body gamma counting with a Beckman Bio-Gamma counter, and counts were adjusted for decay of the isotope during

the course of the experiment. All mice were maintained on water containing 0.10%(w/v) NaI to prevent re-uptake of released ^{131}I by the thyroid.

C. Results

Experimental Design: During the course of other studies, we identified a male C3H/HeJ <-> Balb/cCr tetraparental mouse, which despite an overall chimerism of (Balb 80: C3H 20), showed a pure C3H germ line, as assessed by mating with Balb/cCr females. All (58/58) of the progeny of these test matings were agouti. This animal, which is most likely an XX/XY chimera (McLaren, 1975, 1976), thus afforded us an exceptional opportunity to test the hypothesis that tolerance to H-2 antigens resulting from the chimeric state can be transmitted through the male germ line. He was subsequently mated to 10 different C3H/HeJ females, and the surviving progeny were all tested for tolerance to Balb H-2*d* antigens as detailed below.

To compare the state of tolerance induced in adult animals with that obtained during foetal development, separated DBA/2J parabiont males, which showed stable tolerance to (C3H.HeJ x DBA/2J)F_1 skin (Drell and Wegmann, 1979), were mated to normal DBA/2J female mice. Tolerance to C3H.HeJ MHC antigens in the offspring was assessed by their ability to mount a first set reaction to H-2*k* foetal heart grafts.

Results of heart grafting: The data in Figure 11 show the survival of heart grafts as determined electrocardiographically in age matched control and putatively tolerant ('tol') animals derived from the tetraparental matings. One hundred percent of Balb/cCr controls showed acceptance of Balb heart grafts at 14 days after grafting, while no C3H controls and 1/18 putatively tolerant progeny had functioning grafts at that time. Graft activity in this one animal had disappeared by day 17. Additionally, C3H and C3H('tol') animals did not appear to differ with respect to the kinetics of rejection.

Parabiont mating studies gave comparable results, with similar kinetics of rejection for C3H.HeJ allografts (Figure 12).

Tumour clearance: Balb/cCr animals showed slow clearance of the L1210 leukaemia, consistent with failure of rejection. Indeed, all animals eventually went on to die from the tumour. By contrast, C3H and C3H(tol) animals showed a rapid loss of label, consistent with second set (accelerated) rejection. The slopes of the clearance lines for

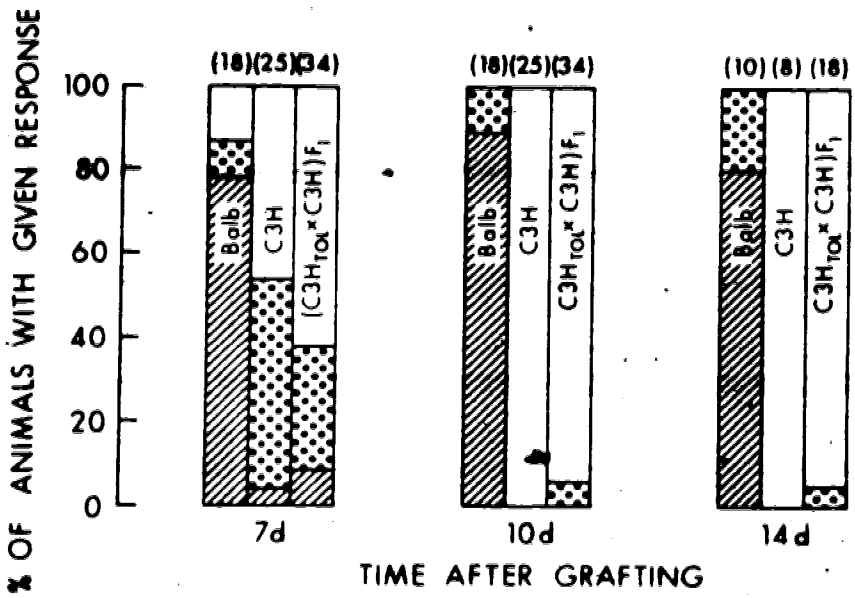

Figure 11: Survival of Balb/cCr foetal heart grafts in control animals and progeny of tetraparental matings. Number of animals in each group is shown in parentheses above each column. Electrical activity in grafts: ■ > 5 uV; ▩ 1 – 5 uV; □ < 1 uV.

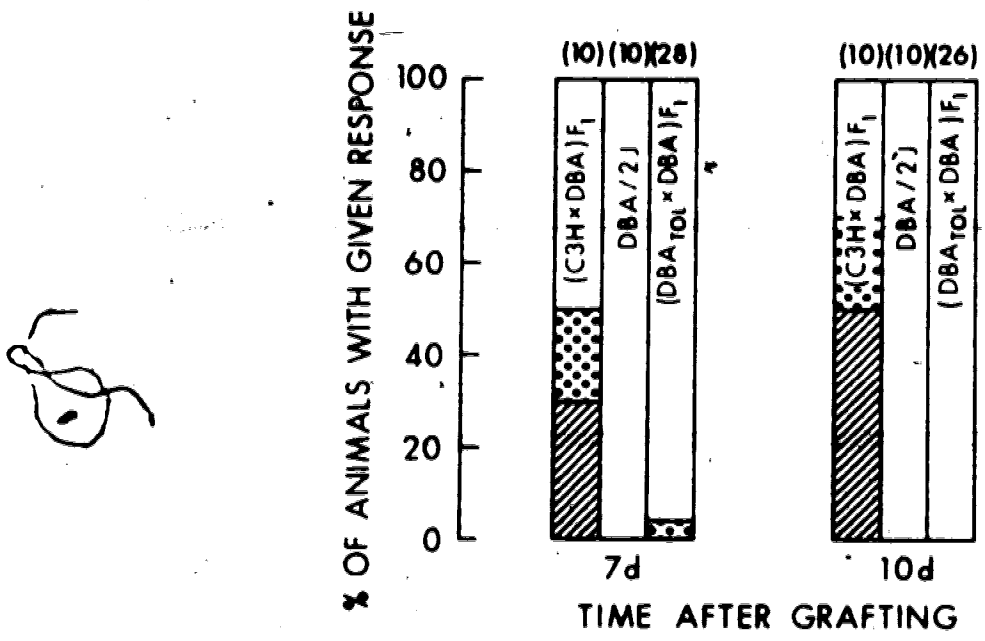

Figure 12 Survival of C3H.HeJ foetal heart grafts in control animals and progeny of parabiont matings. Number of animals in each group is shown in parentheses above each column. Electrical activity in grafts: ▨ > 5 uV; ⊡ 1 – 5 uV; □ < 1 uV.

normal and putatively tolerant animals did not differ from each other (Figure 13), arguing against even partial tolerance to H-2*d* antigens, or a deficit in priming.

Thus, the ability to mount both first and second set allograft responses was not impaired in the progeny of the mating of an tetraparental tolerant male with a number of normal females, as compared to normal syngeneic animals. Similar findings with respect to first set responses were obtained in matings with the parabiont male.

D. Discussion

The work of Gorczynski and Steele (1980, 1981) finds a substantial precedent in the studies of Guttman *et al* (1964), and of Kanazawa and Imai (1974), the former group having also studied the heritability of induced immunological tolerance, and the latter, that of foreign H-2 antigens. Further evidence in support of genetic fixation of environmental influences comes from the classic studies of Evans and his collaborators, who described nuclear changes associated with induced phenotypic changes in the flax variety *Stormont Cirrus* (Evans, 1968).

All of these results are consistent with a Lamarckian interpretation, yet the immunological studies leave unanswered certain key points, chiefly technical aspects of tolerance induction and assay, and the mechanisms by which induced changes become fixed in the germ-line.

Our own studies reported here give a contrary view, namely that the H-2 tolerant state in stable tetraparental mouse chimeras is not heritable, despite the fact that chimerism extends throughout most tissues of the body. None of the progeny of the tetraparental matings were tolerant of Balb/cCr MHC antigens, as measured by prolongation of heart allograft survival. Although 1/18 of the putatively tolerant progeny had a weakly functioning graft at 14 days (but not by 17 days), we do not consider this evidence of significant hyporesponsiveness ($P > 0.1$ by the fourfold table test). Similarly, the kinetics of rejection of the L1210 leukaemia were identical for 'tolerant' and control animals, and were characteristic of second set (accelerated) rejection.

Our parabiont mating studies also clearly show that heritable tolerance cannot be fixed in the germ-line of male animals tolerized to foreign histocompatibility antigens by parabiosis to semiallogeneic partners. No evidence was found amongst these animals for

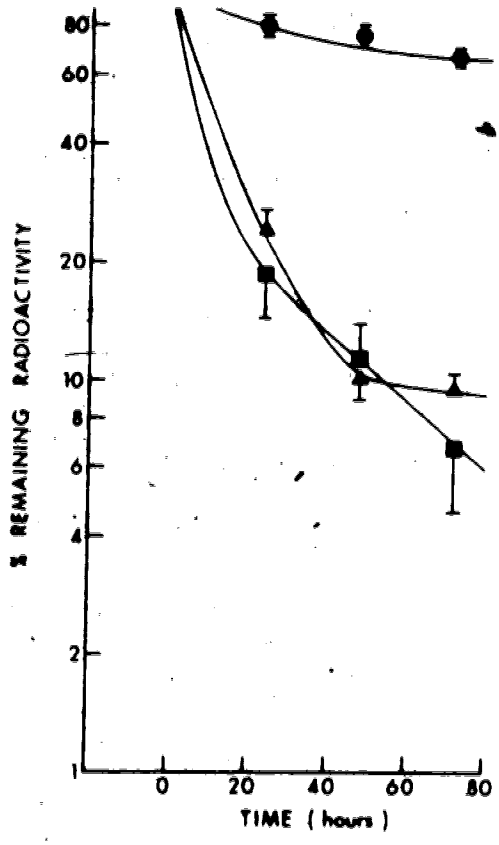

Figure 13: Clearance of ^{131}IUdR-labelled L1210 tumour cells *in vivo* by heart grafted control and tetraparental-progeny animals. ● Balb/cCr controls; ■ C3H.HeJ controls; ▲ tetraparental progeny.

Figure 13: Clearance of ^{131}IUdR-labelled L1210 tumour cells *in vivo* by heart grafted control and tetraparental-progeny animals. Balb/cCr controls; C3HHeJ controls; tetraparental progeny.

variation in the ability of different males to transmit tolerance, as reported initially by Gorczynski and Steele (1980), and claimed by Steele (1981) in the data of Brent *et al* (1981).

It is possible that differences in experimental conditions may account for the discrepancy between our results and those of Gorczynski and Steele. The time relationship for tolerance induction, and perhaps its mechanism, is strikingly different in tetraparental as opposed to neonatally-tolerant animals, and it may be postulated that, unlike the active tolerance of the Billingham and Brent model (1956), the 'passively'-acquired tolerance of the tetraparental mouse is incapable of being fixed in the germ-line. If, as suggested by Steele (1979), environmental pressure is of particular importance in forcing genetic transduction, it may further be supposed that the repeated injections of semiallogeneic cells used by these authors to maintain the chimeric state (Gorczynski and Steele, 1980, 1981) would tend to favor this process. Nonetheless, the work of Guttman *et al* (1964) suggests that a transmissible state of tolerance can be induced by a single injection of tolerizing cells immediately after birth, although this tolerance tended to wane with time. The means by which tolerance is assessed is also of importance. Guttman's studies employed direct *in vivo* assays of skin graft acceptance and tumour take (Guttman *et al*, 1964), while Gorzynski and Steele's work relied on a more indirect criterion, the ability to generate cytotoxic T-lymphocytes against the putatively tolerated H-2 type *in vitro* (1980, 1981), an assay which has been claimed to correlate with *in vivo* graft rejection (Gorczynski *et al*, 1978). In the present paper, we have used *in vivo* assays similar to Guttman's, although more accurately quantifiable, which allow us to discriminate between first-set rejection, and the ability to be primed for accelerated rejection of a second graft. Our results with both assays argue strongly against the possibility of soma -> germline transmission of tolerance, at least in our experimental system.

Recently, Brent *et al* (1981) and McLaren *et al* (1981) have reported results similar to ours, with neonatally-tolerant and tetraparental male mice, respectively.

The Brent paper (1981) is significant in that includes a direct comparison of the *in vitro* CTL and *in vivo* skin graft rejection assays, obtaining concordant and negative results in both systems. It therefore seems unlikely that our failure to reproduce the

Gorczynski and Steele (1980, 1981) results is a reflection of our assay systems. Similarly, McLaren *et al* (1981) found no evidence for vertical transmission of immunological hyporesponsiveness in breeding studies with tetraparental males, assayed by the *in vitro* generation of CTL.

Thus, the work suggesting that acquired tolerance can be transmitted through the male germ-line does not appear to represent a general phenomenon. Our results, together with those in other recent reports, place apparently strict limits on the conditions under which it can be observed, particularly with physiological assay systems.

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