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ROLE OF SIX HOUR T CELLS IN IMMUNITY

by

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TO MY WIFE, SELINA AND MY PARENTS

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RATIONALE

Ever since the discovery that the collaboration of two lymphocyte populations were necessary for the development of humoral antibody responses, data have rapidly accumulated to implicate participation of T cells in many immune phenomena. It also soon became evident that T cells play a dual role in the development of immune responses. On the one hand, T cells have been shown to provide critical 'helper' effects, while on the other hand, T cells have been equally well known to exert negative effects on the development of immunity. These observations generated a number of significant and interesting questions. For example, what are the natures of the helper and inhibitory cells? Do they represent different subpopulations of T cells or are they one cell at different stages of differentiation? What is the mechanism(s) underlying collaboration and initiation of an immune response? Once induced, how is an immune response regulated and terminated?

Previous studies in this laboratory demonstrated that a relatively large subpopulation (25%) of splenic T cells acquired readily detectable surface immunoglobulin within 6 hours following antigenic stimulation (1,2). Complexes of Ig and antigen are present in serum at the same time and have been shown to be cytophilic for T cells in vitro (3). This observation, which represents one of the earliest known phenomena induced in vivo by antigenic stimulation, posed the immediate question as to its functional relevance. Basically, two approaches were adopted to delineate the biological

significance of the observed changes at 6 hours. A direct approach involving the adoptive transfer of 6 hour cells has been undertaken by others in the laboratory.

A different approach is to abrogate the immune response by some tolerizing regime and then observe the effects on the 6 hour phenomenon.

The subject of this thesis represents efforts along the lines of the second approach in an attempt to answer the following questions:

(1) Does the 6 hour phenomenon have a role in humoral antibody formation or cell mediated immunity or both? If so,

(2) does it reflect an inductive or regulatory mechanism?

It is hoped that the results of these efforts will throw some light on the fundamental issues of induction, regulation and termination of the immune response and contribute to a better understanding of the complexities of the immune system.

In addition, preliminary experiments to define the role of suppressive factors in this mode of tolerance induction are also included.

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ABC	antigen binding cell
Ab(s)	antibody (ies)
ALS	anti-lymphocyte serum
$\alpha\theta$	anti-theta antiserum
αMIg-αBSA	hybrid antibody which detects all classes of mouse Ig.
ATS	anti-thymocyte serum
B cells	bone marrow derived lymphocytes
BCG	Bacillus Calmette-Guerin
BDB	Bis-diazotized benzidine
BSA	bovine serum albumin
CMC	cell-mediated cytotoxicity
CNBr	cyanogen bromide
Con A	concanavalin A
CRBC	chicken red blood cells
CRL	complement receptor carrying lymphocytes
DEAE	diethylaminoethyl
DTH	delayed type hypersensitivity
EA	chicken egg albumin
FCA	Freund's complete adjuvant
FCS	fetal calf serum
Fe	ferritin
FIB	human fibrinogen
HBSS	Hank's balanced salt solution

HHS	horse erythrocyte hemolysate supernatant
HRBC	horse red blood cells
Ig	immunoglobulin
i.p.	intraperitoneally
KLH	keyhole limpet hemocyanin
MBLA	mouse specific bone marrow derived lymphocyte antigen
MHC	major histocompatibility complex
MSLA	mouse specific lymphocyte antigen
NRS	normal rabbit serum
PFC	plaque forming cell
PHA	phytohemagglutinin
POL	polymerized flagellin
RFC	rosette forming cell
RICA	reverse immunocytoadherence
R.T.	room temperature
SIII	pneumococcal polysaccharide type III
SHS	sheep erythrocyte hemolysate supernatant
SRBC	sheep red blood cells
T cells	thymus derived lymphocytes
T-RFC	T-rosette forming cell
θ	theta isoantigen

ABSTRACT

Investigations have been undertaken in our laboratory to study the early effects of antigen on B and T cells in spleens of mice during a primary response to antigen. The reverse immunocytoadherence technique (RICA) was used to detect surface associated immunoglobulin on mouse lymphocytes. The technique involves the use of a 5s hybrid antibody which has an anti-mouse Ig site and an antiprotein site. A rosette is formed as a result of the reaction between the anti-Ig site with surface associated Ig and the antiprotein site with protein coated sheep red cells. Utilizing RICA and an antitheta antiserum-complement cytotoxicity assay, it has been demonstrated that 25% of splenic T cells acquired easily demonstrable surface Ig 6 hours after primary immunization. Concomitantly, there is a comparable decrease in the level of theta carrying lymphocytes. Furthermore, cytophilic complexes of antigen with immunoglobulin mediated by a soluble factor have also been detected in sera of immune animals.

This thesis studies the effects of antigen upon B and T cells in animals rendered immunologically unresponsive to HRBC, and evaluates the effects of tolerance upon the serum factors and cytophilic complexes identified during a normal primary response.

Mice, made unresponsive to HRBC by repeated injections of an ultracentrifuged horse red blood cell hemolysate preparation, were challenged with an immunogenic dose of HRBC

and their spleen cells analyzed 6 hours after challenge. In contrast to normal mice, T cells in these animals did not acquire surface immunoglobulin as detected by RICA. Similarly no 6 hour Ig carrying T cells were observed upon immunizing tolerant mice with a battery of heterologous antigens. Experiments further revealed that tolerant spleen cells were unable to pick up preformed homologous or heterologous cytophilic complexes reflecting some deficit in their membrane properties. However, six hour sera collected from tolerant mice immunized with particulate antigens only contained cytophilic complexes. Whether these complexes are quantitatively and qualitatively similar to those obtained from normal mice is uncertain at the present time. Although the 6 hour T cells appear not to be directly involved in the specific induction of humoral responses, it is quite possible that they may be concerned with the induction of other aspects of the immune response and/or with immunoregulation. Results of experiments reported here suggested that the 6 hour T cell may well play some role in regulating T cell mediated immune functions such as delayed type hypersensitive reactions, cytotoxic effector lymphocyte activity and DNA synthetic responses to plant mitogens. Other results implicate the 6 hour T cell in an amplifying role in antibody formation. Thus, 6 hour immune spleen cells obtained from normal animals immunized with SRBC were capable of specifically augmenting the antibody response to SRBC in an irradiated recipient. On the other hand, six hour spleen cells from SRBC immunized

HRBC tolerant animals, which have been shown to lack Ig+ T cells failed to show any enhancing capabilities. Nevertheless, they were fully capable of supporting a normal anti SRBC response comparable to that given by nonprimed normal spleen cells. These findings strongly suggest that the 6 hour T cells and the more conventionally known Ig- 'helper T cells' are two distinct subpopulation of primed T cells. The hypothesis is proposed according to which the 6 hour T cell probably subserves a regulatory role and amplifies the basic helper T function by some as yet undetermined mechanism.

The possible role of suppressive factors mediating this particular type of tolerance was also investigated. Preliminary data, although suggestive of the presence of suppressive activity in tolerant serum are far from conclusive and have not established any strict correlation between these suppressive factors and tolerance induction or maintainance.

LITERATURE REVIEW

I. IMMUNOLOGICAL CONCEPTS: A BRIEF HISTORY

Long before Immunology became a science in its own rights, it was well recognized that persons who recovered from an infectious disease remained immune for some time, if not for life, to reinfection. Thus, the ancient Chinese sniffed dried powders of smallpox crusts to obtain immunity without inducing suffering from the actual disease and the inhabitants of Circassia innoculated their infants with pustules from smallpox lesions. These efforts represent the first known examples of artificial active immunization and demonstrated clearly the concept that immunity may be acquired by previous exposure to the immunizing agent.

In 1798 Edward Jenner discovered the basis of the modern method of vaccination when he successfully conferred protection against the more dangerous smallpox by innoculating human beings with fluid from a cowpox sore.

About 100 years later, Louis Pasteur made the fortuitous observation that innoculation of chickens with old cultures of cholera provided them with immunity against cultures of the virulent form of the microbe. Shortly afterwards, in 1881 he succeeded in attenuating the virulence of anthrax bacillus and in conferring immunity against anthrax in sheep and goats injected with the attenuated bacillus. In 1882 he produced a vaccine for the protection of human beings and dogs against rabies.

Up to this time, the phenomenon of immunity was looked upon at the level of whole animals, but it was soon

recognized that some blood borne agents were responsible for affording protection against infectious diseases. Buchner in 1889 and Pfeiffer in 1894 demonstrated the innate capacity of blood to kill bacteria; von Behring and Kitasato in 1890 showed that the serum of animals injected with diptheria toxin was capable of neutralizing the toxicity of the toxin and that this property was transferrable to another animal. This is the first example of passive immunization. Moreover, it was soon discovered that antitoxin could be developed against toxins other than those of bacterial origin, as exemplified by Ehrlich's experiments with ricin, a poisonous extract of castor oil. Continued interest in immune sera soon led to the discovery of other properties of the 'antibody' such as agglutination (1889 Charin and Roger) and precipitation (1897 Kraus), as well as the bactericidal components now known as the complement system (1893 Buchner and 1900 Bordet).

Concurrent with the widely accepted humoral theories of immunity an alternate way of looking at the defence against bacteria as a function of the white cells of the blood was developing. Thus Metchnikoff in 1884 observed that the mobile cells of starfish larva in fluid could engulf solid particles. He later showed that the leukocytes of blood could also engulf and destroy disease producing bacteria. He coined the term 'phagocytosis' to describe the process of engulfment and named these cells phagocytes thereby introducing the concept of cellular immunity.

Theories regarding opsonins gradually brought recognition of the complexity of the humoral factors involved in the immune response. At the same time Bordet's recognition of immune lysis of foreign red cells in 1898 and Landsteiner's discovery of the ABO blood groups in 1904, together with Ehrlich's demonstration in 1901 that goats would produce antibodies against red cells of other goats but not against their own led to the realization of the self vs non-self or 'foreign' concept and the appreciation of antigenic individuality.

A number of observations made at about the beginning of this century by Richet, Portier and von Pirquet indicated that similar mechanisms to those involved in protection from microbial infection could produce harmful effects, such as tissue damage. Thus, the terms 'hypersensitivity' and 'allergy' were introduced to described the increased and altered immunological reactivity of a wide range of tissues on re-exposure to the same agent.

Rapid advances in protein chemistry resulted in a new turning point in understanding immunologic mechanisms. Tiselius and Kabat opened up new fields of immunochemistry in 1938 when they studied the migration of human serum proteins in an electric field. Their discovery that the antibody activity of immune serum was in fact found only in the γ-globulin fraction enabled more precise quantitative studies of antigen-antibody reactions. The source of these antibodies next became the prime target of

research. Studies by Fagraeus in 1948 and Coons in 1955 implicated the plasma cells and lymphocytes in the production of γ-globulins. In 1960 Good and colleagues in Minnesota and Miller in England independently established the essential role of the thymus gland in immunity. The significance of the bursa in chickens in the production of antibody was also brought to light in 1957 by Glick. To accomodate these new discoveries, Burnet in 1959 (4) postulated the Clonal Selection Theory which advocates a one cell - one antibody hypothesis. According to this theory, many different kinds of clones of cells, capable of recognizing different antigen develop spontaneously by the process of random somatic mutation. Antigen will selectively react with the specific clone via receptors and induce further differentiation of the cell to produce a specific antibody. During the process of random mutation, a clone of cells (forbidden clone) which will react with one's own tissue antigen may develop. Such clones are rapidly eliminated by early contact with self antigens before they can develop further (forbidden clone). Although the theory explains how an immune response takes place following immunization and the phenomenon of self tolerance as well as the pathogenesis of autoimmune disease, it is unable to explain how the genome of an individual could contain sufficient genetic information to code for the production of the enormous variety of antibodies theoretically possible and how an individual cell express only one or

two of these possibilities.

In summary, these seemingly unconnected phenomena provided the foundation on which modern immunology has been built, for they all contain as a common denominator the tissue reactions of animals (and man) to foreign cells or substances (i.e. non-self constituents) that enter the body. These tissue reactions which constitute the immune response can be beneficial or harmful as seen respectively in the fields of immunity to infectious diseases and allergy.

Immunology today, therefore, is a science concerned with the study of the basic molecular and cellular factors underlying the process of recognition of self and non-self; the mechanism and regulation of the processes of antibody formation and of cell mediated immunity; the physiochemical nature of antigens and antibodies and the laws governing their interactions; the study of in vitro correlates of cell mediated immunity as well as the humoral and cellular manifestations of the overall immune processes in the tissues of man and higher animals.

In order to realize these objectives, a basic knowledge of the participating immunologic agents and a clear understanding of their interactions is of paramount importance.

The following sections briefly review some of the major components of the immune apparatus, their physical and functional characteristics, their ability to recognize and respond to antigen and their interactions in the production of various immune phenomena.

II. CELLULAR COMPONENTS OF THE IMMUNE SYSTEM

1. Hemapoietic Stem Cells

Stem cells are ancestral, non specialized cells with the potential for extensive proliferation, self renewal and differentiation to more mature forms (5).

Initially derived from hemangioblast cells residing in the blood islets of the yolk sac, and later found in fetal liver, hemapoietic stem cells are found chiefly in the bone marrow in the adult although cells of similar function exist in the thymus, spleen and avian bursa of Fabricius (6-8).

These stem cells are seeded via the blood stream into various hemopoietic tissues where their differentiation is channelled exclusively to either lymphoid or myeloid pathways (7,9,10), the direction of differentiation being specified according to the inductive influence provided by the microenvironment into which the stem cells have migrated (6,7,11). The nature of these inductive forces is still a mystery although soluble factors such as erythropoietin (12) or thymus humoral factors may be involved (13-15).

2. Primary Lymphoid Organs

The primary lymphoid organs consisting of the thymus and the bursa of Fabricius are lymphoepithelial organs, are derived from ectoendodermal functions in association with the gut epithelium (16) and are sites of

differentiation of antigen reactive or immunocompetent cells (17,18).

i. The Thymus

In mice, studies on the repopulation of the thymus following irradiation and on the regeneration of thymus grafts have established that lymphoid cells repopulating the epithelial cytoreticulum of the thymus are derived from a circulating cell, the origin of which can be traced to the fetal liver or bone marrow (19,20). In man, the thymus is generated from the epithelium of the third and fourth pharyngeal pouches at about the sixth week of gestation (21,22). Mesodermal elements start to aggregate around the epithelium and by the eighth week, the thymus is differentiated into a compact epithelial structure. Pluripotent stem cells originating in bone marrow (23) find their way via blood stream to the thymus, and mature into immunologically competent cells. The work of Trainin et al (14) and Goldstein et al (15) implicates a thymic hormone in the maturation of stem cells into immunocompetent cells in the thymic microenvironment. Studies using the incorporation of H^3 - thymidine show that the thymus is engaged actively in lymphopoiesis, in fact it has a higher rate than any other lymphoid organ (24). The reason for this intense lymphopoiesis, however, is still unknown and unlike lymphopoiesis in the secondary lymphoid organs, thymic lymphopoiesis is independent of antigenic stimulation. Post-thymic

cells which amount to a small fraction of thymocytes, after developing full immunologic competence, circulate through blood and lymph and tend to reside in specialized regions of bird or mammalian peripheral lymphoid tissues (25-30). The major proportion of thymocytes, however, die locally within the thymus without subserving any obvious immunological function (31). The purpose of this local birth and death is still a matter for conjecture.

ii. The Bursa of Fabricius and Possible Mammalian Analogs

The Bursa of Fabricius, a lymphoepithelial organ so far found only in birds develops from the dorsal part of the urodeal-proctodeal plate forming the cloaca. Numerous epithelial buds appear as early as the 12th day of embryonic life followed a few days later by the development of large follicles containing lymphocytes in these birds (16). Similar to the thymus, lymphopoiesis is intense and apparently independent of antigenic stimulation. The bursa appears to provide a special microenvironment for the incoming lymphoid stem cells to differentiate and mature into immunocompetent cells (6). These lymphocytes then migrate to specified regions of the lymph node and the spleen (29, 32-35). In mammals these thymus independent areas are found in the far cortical areas and medullary cords of the lymph node and the mantle, germinal follicles, and perifollicular regions of the spleen (33-36).

In mammals, the search for distinct lymphoid entity

analogous to the bursa in chickens has not been successful. Good and his colleagues first suggested that the rabbit appendix was a bursal equivalent (37) but this was not supported by the finding that neonatal thymectomy and appendectomy in rabbits did not inhibit the development of plasma cells and immunoglobulins (38). Later the tonsils were considered as possible candidates (39) and more recently, evidence was given that Peyer's patches were bursal analogues in rabbits (40). This latter view, however, was inconsistent with the fact that Peyer's patches exhibited characteristic features of secondary rather than primary lymphoid tissues (41-43). It is possible that a single or multiple organ with specifically restricted bursal function need not exist as such in mammals (17,44) but that the gut epithelium might produce a humoral factor that specifies the differentiation of antibody forming potential in cells populating the lymphoid follicles dispersed throughout the secondary lymphoid tissues, including the appendix and the Peyer's patches.

3. Secondary Lymphoid Organs

Peripheral lymphoid organs comprise of lymph nodes, spleen, Peyer's patches, pharyngeal and palatine tonsils, appendix and milky spots of the omentum. Whether or not some of these tissues also exert a bursa-like inductive function (38,40-44), all are sites predominantly of antigen driven division and cellular differentiation with

well developed antigen-trapping and lymph filtration mechanism (45-48). In addition, the generation of the immunologically activated cells mediating immune responses occurs in the secondary lymphoid tissues (45). It is also well-documented that there exist , in secondary lymphoid tissues notably the spleen and lymph node, anatomically distinct areas to which lymphocytes of thymic and marrow or bursal origin will preferrentially 'home' (29,32,49-54). Thus the periarteriolar sheath of the splenic Malpighian body, the mid and deep cortex of the lymph node and the inter-nodular space of gut associated lymphoid organs are termed thymus dependent areas (29,49,53) while remaining areas consisting of nodules and medulla in lymph node, nodules and plasma cells in the gut-associated lymphoid organs, the peripheral layer of the Malpighian body in the spleen, the perifollicular area and the red pulp were all designated thymus-independent or marrow dependent (49, 53-55).

4. Immunocompetent Cell

i. Functional Characteristics

Immunological responses are classically divided into those mediated by humoral antibody and those by cells. Both depend upon the activity of immunologically competent small lymphocytes ultimately derived from precursors in the bone marrow (5,56). These cells,

although not actually engaged in an immune response, are nevertheless fully capable of initiating such responses when appropriately stimulated by antigen (57). Thus immunocompetent lymphocytes can be divided, according to their functional differences into two major types:

(1) Thymus or thymus derived cells (T) responsible for cell mediated reactions such as homograft rejection and delayed-type hypersensitivity and also humoral antibody response to certain antigens (45).

(2) Bone marrow or bone marrow derived cells (bursal or bursal equivalent derived cells, B) which are precursors of plasma cells responsible for the production of humoral antibody (5,58,59).

T and B cells also differ in their distribution (previously discussed) and probably in their life span; the T lymphocytes constitute the greater part of the recirculating pool of small lymphocytes and have a relatively long life (5,58) while the B lymphocytes are more restricted to lymphoid tissue and appear to be short-lived (27).

A. Thymus Derived Lymphocytes

In mice, neonatal thymectomy results in marked depression of cell-mediated immunologic reactions such as homograft rejection and delayed hypersensitivity reactions as well as some antibody responses (60). This could be restored either by thymic grafts or by grafts enclosed in a cell-impermeable Millipore diffusion chamber (13,62).

A similar effect of neonatal thymectomy was also found in the chicken (18). Although thymus derived cells are required in the development of antibody responses to protein and erythrocyte antigens (63,64), humoral responses to antigens such as hemocyanin and pneumococcal polysaccharide (34) remained intact in their absence.

The existence of disease states such as the sex-linked recessive agammaglobulinaemia of Bruton (65), the Swiss type of agammaglobulinaemia and alymphocytosis (66), and DiGeorge Syndrome (67,68) suggest that in man, too, the thymus is important in the development of cell mediated immunity. On the other hand, Davies has shown that although thymus-derived cells proliferate in response to stimulation by various antigens (69), they do not themselves differentiate into antibody forming cells (19,29).

Important as the thymus may be in terms of the immunological integrity of an animal, it was found that thymocytes themselves are non-immunocompetent as compared to lymph node or spleen cells (70-72). Consequently the thymus is regarded as a primary organ not itself engaged in immune responses but required for correct development of the secondary lymphoid organs.

B. Bone Marrow Derived Lymphocytes

In considering the heterogeneous nature of specific immune responses, studies of chicken lymphoid system first indicated the existence of two distinct lymphoid cell differentiation pathways that were respectively concerned

with cell mediated and humoral immunity. The pioneering studies of Glick et al (73) showed that neonatal removal of the avian bursa of Fabricius considerably depressed the subsequent ability of the bird to produce specific antibodies upon immunization. These observations were extended by Mueller et al (74) and confirmed by studies from the Hall Institute (75,76) showing that hormonally bursectomized chickens failed to form humoral antibodies but maintained their power to reject skin allografts. Work by Toivanen et al (77) showed that the restoration of the ability of bursectomized chickens to produce antibodies can be corrected by the transplantation of syngeneic bursal cells from young chickens. Although antibody producing cells are absent in the bursa (78), these cells have the inherent capacity to transfer antibody production to irradiated birds (79) thus suggesting that this organ contains precursors of antibody forming cells. Like the thymus then, the avian bursa is considered to be a primary lymphoid organ responsible for providing the precursor cells which ultimately are responsible for the effects of humoral immunity.

As mentioned previously, no bursal analog per se has been found in mammals but it is possible that the gut epithelium produces some factor that controls the differentiation of antibody forming precursor cells (80, 81). It has been widely accepted that the adult bone marrow serves as the chief source of stem cells in the animal (82,83) and also as the bursal equivalent as

previously discussed.

C. Accessory Cells

At a more peripheral level than the direct specific interaction of an antigen with immunocompetent cells are various other cell types that play accessory roles either in the early phase of antigen handling or at the effector level. Thus, cells of the reticuloendothelial systems, macrophages, monocytes and dendritic reticulum cells in lymphoid follicles play various roles in the development of some (84) but not all (85) humoral antibody responses; whereas mast cells, for example, play an accessory effector role in certain hypersensitivity responses (86, 87).

ii. Structural Characteristics

Bone marrow derived (B) and thymus derived (T) lymphocytes differ not only in function but are characterized by distinct surface markers. These differences are extremely helpful in their identification and separation. The discussion which follows is a brief overview on the better characterized and more commonly used surface markers of the two lymphoid populations.

A. Bone Marrow Derived Cells

(a) Surface Ig

Surface immunoglobulin has to be the most well known and widely utilized marker for B cells. Various direct techniques have been employed to demonstrate the presence of immunoglobulin on the surface of B cells. Amongst them,

immunofluorescence utilizing fluorescin and rhodamine labelled anti-immunoglobulin (88,89) and autoradiography using radioactively iodinated (90-93) anti-immunoglobulin antibodies have enjoyed popular usage. Other techniques such as the mixed agglutination technique (94,95) and the reverse immune cytoadherence technique which utilizes a 5s hybrid antibody and rosette formation (96-98) have also been used. That these surface immunoglobulin are an integral part of the cell membrane and are not physically adsorbed has also been demonstrated (99,100).

Many studies, using a variety of animal species, have confirmed that B cells are the principal cell type that expresses high density surface immunoglobulin. Thus Klein et al (101) and Abdou and Abdou (102) showed that the bone marrow in man contains a population of cells bearing surface IgM and that these cells are likely to be of the non thymic derived B cell series. The use of other characteristic T and B cell markers in combination or alone clearly showed that cells bearing readily demonstrable surface Ig do not carry T cell markers but frequently carry other B cell markers and that high density surface Ig is a property of B cells (103-106). Similar findings were obtained from studies involving mice (88,90-92,107-113), rabbit (114-120, 159), chicken (121-125), rat (126,127) and guinea pigs (116, 128-131).

Mature antibody-forming plasma cells are clearly restricted in their expression of Ig structural genes, with most of the cells producing only one type of L-chain and

H-chain gene product at both the class and allotypic levels. Immunofluorescent studies of Ig-containing plasma cells from heterozygous rabbits revealed only one H-chain and one L-chain type per cell (132,133). By developing Jerne plagues to sheep erythrocytes with plasma cells from heterozygous or homozygous rabbits, Chou et al (134) showed that only one L-chain allele was expressed per cell in the heterozygote. In addition these workers gave data indicating that the same applies in homozygous animals. Restriction of Ig, using electrophoretic analysis of proteins from single antibody forming cell, has shown homogeneity comparable to that obtained with single myeloma cells (135). Analysis of the class of antibody made by single cells has revealed that over 95% of the cells produce specific antibody of only one H-chain class (136-140). Nevertheless, examination of Ig classes and allotypes on the cell surface of B lymphocytes using polyvalent anti-Ig reagents and class specific antisera, has demonstrated that a sizeable portion of B lymphocytes display multiple heavy chain classes on their surface (92,97,98,119,137-150). On the other hand, an equally formidable amount of data exists to support the opposite view. Thus it was shown that only one heavy chain class exists on surface of B cells in humans (117,151-159), rabbits (89,117,118,120), mice (91,111,112,160) and chicken (121-123).

It must be concluded from the number of studies that failed to show cells exhibiting multiple Ig class or

allotype that allelic and class restriction applies to the majority of B lymphocytes. Where doubly labelled cells are found or inferred from population studies, there is always the possibility that it is due to acquisition of Ig from the serum or extra cellular fluid as has been clearly shown to be the case for rabbit allotypes (119). However, the possibility that a small proportion of B cells do express multiple allelic product cannot be completely ignored.

There is a lack of agreement between different laboratories on the predominant molecular class of Ig on B lymphocytes. Studies on human peripheral blood leukocytes evidence seems to favor IgM as the major Ig class on B cells although there are good results to implicate IgG as the dominant surface Ig exposed on B cell surface (161). In recent years, however, it became evident that IgD is yet another major class of surface Ig found in mature B cells in man, monkeys and mice (870). In mice most studies indicate that the majority of cells bear IgM (162-164) while a few showed that 20-45% of peripheral blood lymphocytes carried IgG type surface Ig whereas only 4-25% carried the IgM type (111,165,166). Still others (91,95) have reported that IgM carrying lymphocytes are a relatively minor proportion of Ig+ lymphocytes, with many cells showing IgG or IgA on their surface. Pernis et al (89,117) have shown that about 90% of the Ig bearing cells in rabbits have IgM. Limited information is available on distribution of Ig bearing cells in other animal species, but in general it is likely that IgM is the predominant surface Ig on B lymphocytes.

(b) Complement Receptors

Another membrane marker, identified in B lymphocytes and operationally defined as a complement receptor has been described on normal lymphocytes from many animal species (167-170), in human and guinea pig leukemic cells (129,104,171,172) and in human cell lines (171). It is probably a protein as it is destroyed by trypsin treatment (168)and is probably not membrane bound Ig (171,173,174). The complement component involved has been studied and C3 has been strongly implicated (104,168,175-178).

Complement receptor lymphocytes (CRL) are rare in the thymus but are found frequently in large numbers in the so called thymus independent areas of peripheral lymphoid tissues (51,179). These findings suggests that CRL may be closely correlated with B lymphocytes. More direct evidence has been obtained when Bianco et al (168) showed that most mouse CRL have Ig on their membranes, whereas non CRL do not. Also, passage of mouse spleen cell suspensions through anti-Ig coated columns, a method which reportedly binds B cells selectively, depletesthe cell population of CRL (113). In addition, mouse CRL do not bear the theta antigen, a T cell marker, on their surface (173) and cell transfer experiments indicate that they are derived from the bone marrow and do not require the thymus for their development (180). Among guinea pig lymph node cells (129) and human tonsilar lymphocytes (181), those cells with surface Ig as detected by immunofluorescence or autoradiography also form

rosettes with erythrocyte-antierythrocyte-complement complexes.

(c) Fc Receptors

Lymphocytes from several animal species including man have receptors for Fc fragments of antibody or for antigen-antibody complexes prepared in the absence of complement (182-187). The Fc receptor is detected either by a radioautographic technique in which lymphoid cells are incubated with antibody (183) or by the interference of detection of surface Ig (98,186). In normal mice, lymphocytes that bind the complexes through the Ig receptor are B lymphocytes. They belong to the CRL population (188), do not bear the θ antigen, constitute the predominant population of cells in the thoracic duct of nude athymic mice (183) and bear Ig on their membranes (186). In addition, it has been shown that all human peripheral blood lymphocytes that bind aggregated Ig also have membrane Ig. A number of investigations designed to test the possibility that the Fc receptor and membrane bound Ig are one and the same indicated that this is not likely the case. Thus the receptor is not destroyed by trypsin (159), an enzyme treatment known to digest Ig from membrane of B lymphocytes and pre-treatment of cells with anti-Ig did not modify the binding of Ag-Ab complexes (183). Also, it has been shown with human leukemic cells that polar concentration or cap formation of cell surface Ig determinants could be induced but that these caps of Ig could be dispersed upon

subsequent incubation with aggregated Ig (189). The general conclusion was that the receptor for aggregated Ig or Ab-Ag complexes is not the membrane bound Ig. Evidence has also been accumulated in support of the contention that Fc receptors are distinquishable, at least operationally, from membrane receptors binding Ag-Ab-complement complexes (188).

Some investigators suggest that the phospholipids of cell membranes may be the Fc receptors (190,871,872). This hypothesis was supported by the trypsin insensitivity of the receptor as well as by the observation that the receptors for cytophilic antibodies on macrophages are destroyed by phospholipase A (191). However, there is now conclusive evidence that the Fc receptor is a protein or glycoprotein(873).

(d) Mouse Specific Bone Marrow Derived Lymphocyte Antigen (MBLA)

Finally, a less common B cell marker not found on thymus derived cells has also been documented and designated as mouse specific bone marrow derived lymphocyte antigen (112,192,193). However, to date, relatively little is known about this marker.

B. Thymus Derived Cells

(a) Surface Ig

From the previous considerations, it appears evident that B cells but not T cells bear high density surface Ig. However, the presence of surface Ig on T cells is extremely controversial. Thus some investigators found little or no Ig on the surface of T cells (88,89,91,95,112,127,192,160, 194-201). Others, in contrast, reported that T cells

exhibit as much surface Ig as B lymphocytes (163,164, 202-208). Several studies have indicated that when methods of increasing sensitivity are applied, a higher proportion of thymus cells show membrane bound Ig. Thus, in the radioautographic studies of Bankhurst and Warner (92) and Jones et al (111) up to 6% of labelled thymus cells were found and when the methods were pressed to further sensitivity, the number increased to around 15% of thymus cells (209). With an indirect sandwich method, around 50% thymus cells showed specific labelling with anti-Ig and possibly even all cells (109) gave some degree of binding of anti-Ig. A number of workers claimed that surface Ig is more abundantly found on activated T cells (2,109,163, 198,209-213). Although there are indications that mitogen stimulated T cells show an increase in membrane bound Ig (214,215), other studies did not confirm these observations (196,216,217). Furthermore, these latter studies clearly showed that antigen activated but not mitogen activated T cells express detectable membrane bound Ig (2).

The complexity of the issue is further enhanced when one considers the possibility that the Ig+ cells found in a T cell population may, in reality, be contaminating B cells (104,183,218) or T cells that have acquired cytophilic Ig via an Fc receptor (2,186,213,219,220). Other workers, however, have maintained that the Ig found on T cells are intrinscally synthesized (163,202-204,207).

In view of the large body of conflicting data, it is

evident that the existence, nature or amount of T cell membrane bound Ig is far from resolution. However, from the existing available data, some tentative conclusions could be drawn. Thus it is reasonably safe to assume that most thymus cell preparations contain a small proportion of B cells that contribute to the total surface Ig detected in the population. Also, many if not all, of the bulk of θ-positive T cells likely bear some, albeit varying amounts, of surface Ig which is probably cytophilic in nature.

(b) Fc Receptors

The presence of a receptor for the Fc portion of Ig, formerly thought to be only present on B lymphocytes (183, 184) have now been detected on subpopulations of T cells (2,213,220-227). Here again, the nature of this receptor and its physiologic significance remains unclear. Several groups have recently reported that the Fc receptor appears on T lymphocytes activated by exposure to Ag (213,220,224, 228). Experiments by Stout and Herzenberg, however, led them to conclude that the state of activation and the presence of the Fc receptor on T lymphocytes are not strictly correlated and that the Fc receptor is unlikely to be a general marker for primed T cells but that it may be a marker for differentiation in subsets of T cells other than the cooperator T cell (227).

(c) Theta-alloantigen

The classical T cell marker, θ or Thy-1 was first

described by Reif and Allen in 1963 (229). These workers found that this antigen is under the control of a single locus with two alleles, the θ-AKR in AKR, RF and a few related substrains and the θ-C3H found in most inbred strains of mice (230). Eventually AKR anti-C3Hθ and C3H anti-AKRθ antisera were produced and found to be specific for the θ antigen (193). Utilizing these antisera, Aoki and others demonstrated that thymocytes have more θ on their surface than do peripheral lymphocytes (231,232). The thymus dependence of peripheral θ+ lymphocytes has been shown by the fact that it is markedly depleted in mice which have been treated with ALS (233,234) or which have been thymectomized (235,236) or are congenitally athymic (236). These treated mice are all known to be depleted of lymphocytes in thymus dependent areas (29,237,238).

(d) Mouse Specific Lymphocyte Antigen (MSLA)

Another group of surface antigens, the mouse specific lymphocyte antigen or MSLA found exclusively on thymus derived cells but not bone marrow derived cells have been reported (112,192,239) but have not been widely adopted as a marker for T cells.

III. IMMUNE RECOGNITION

An absolute pre-requisite for the initiation of an immune response is the recognition of an interaction with the antigen by the immunocompetent cell. It would be appropriate, therefore, to discuss at this time some of

the more relevant aspects of immune recognition. These include the existence and nature of antigen binding cells (antigen reactive cells), the nature of antigen receptors and the mechanism of antigen binding.

1. Antigen Binding Cells

A number of techniques have been developed to establish the existence of antigen binding lymphoid cells and to determine the nature of antigen recognition sites on the surface of these cells.

i. Rosette Formation

The direct adherence of large particulate antigens to the surface of lymphoid cells from immunized animals has for years been recognized as a means of enumerating the numbers of antigen binding cells. These studies have primarily used bacteria (240-242) or red cells (243,244); the latter technique is better known as immunocytoadherence or rosette formation. Rosette forming cells are also found to exist in lymphoid tissues of unimmunized animals (245). By coating the surface of erythrocytes with soluble antigens, the rosette technique could be modified to demonstrate the existence of antigen binding cells for a number of antigens in addition to red cell antigens. This was achieved for protein antigens (246-248) and haptenic determinants (249).

ii. Autoradiographic and Fluorescent Techniques

Studies of soluble antigens were also facilitated by the use of radioisotopes or fluorescent labels. Naor and Sulitzeanu (250) were the first to adopt the isotopic approach to quantitate the number of antigen binding cells in normal animals. These studies were then extended by several other teams of investigators (251-256).

The use of fluorescein labelled antigens to detect antigen binding cells (257,258) has been rather limited but the combined use of an automated fluorescence-activated cell sorter (259) has made this a practical marker for screening large numbers of cells and for obtaining these cells for further analysis.

iii. Enzymatic Methods

Enzymatic techniques of detecting antigen binding cells are of two types, the differences in methodology depending on the substrate used: the fluorogenic substrate, fluorescein-di-β-galactoside (260,261); an indigogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (262); or riboflavin galactoside with bacterial colony growth of an anxotrophic mutant requiring riboflavin (263). This concept of binding of antigen that are enzymes followed by detection with substrate has been adapted to other haptenic antigens conjugated to the enzyme.

iv. Solid Phase Methods

By passing cell suspensions through an antigen-coated glass bead column, Wigzell and Anderson (100)were able to deplete selectively specific antigen reactive cells. Inhibition studies with hapten or anti-Ig clearly demonstrated the specificity of retained cells (264). Subsequently, other workers using small ligands attached covalently to large polyacrylamide beads (265-267) developed affinity columns whereby specific antigen reactive cells can not only be bound, but can then be removed by hapten elution, thus providing considerably enriched populations of antigen binding cells. Another modification of the solid phase method has utilized, as supporting medium for antigen coating, taut monofilament nylon fibers (268). Cells are adsorbed to fibers covalently coupled to antigens or lectins and can then be removed by mechanical plucking of the taut fibers.

These techniques described above have not only provided evidence for existence of specific antigen binding cells but have also resulted in a number of meaningful observations critical to the understanding of the whole process of the immune response. Thus it was observed that for a wide range of antigens, with only a few exceptions (187,255,269-271), the values generally show around 0.002 to 0.07% of cells binding antigen (161) which amounts to an average of 25,000 ABC per mouse spleen. Since not all cells that bind antigen in vivo are activated, the above figures probably

represents an overestimation. The actual values are therefore clearly consistent with clonal selection concepts that a small population of cells are responsive to antigen in vivo (250,251,261,272). It was evident that the degree of antigen uptake varies with different antigens as well as for different animal species (251,273). This may reflect variation in absolute numbers of immunocompetent cells (274) or differences in avidity of antigen binding cells for that antigen. Morphologically, these cells are similar to lymphocytes (251,275-284) and binding of antigen is immunologically specific (249,251,272,285,286).

There is ample evidence to support the contention that the major proportion of antigen binding cells are B cells. For example, the absence of antigen binding cells in X-linked agammaglobulinemia (287,288), their presence in congenitally athymic nude mice (273) and in human bone marrow (102); their insensitivity to anti-θ-serum treatment (253); their antigenic origin in avian bursa of Fabricius (289); identical sedimentation velocity distribution profile of antigen binding cells and antibody forming cells (290); inhibition of hapten-carrier binding antigen binding cells by free hapten (252) represent some indirect evidence. Direct evidence includes labelling of an antigen binding cells by fluorescent-anti-Ig (252,112) and the observations that antigen binding cells are MBLA-positive and MSLA-negative (112). The experiments of Julius et al (259) also showed that purified antigen binding cells are antibody-

forming cell precursors. The recognition of antigen by T cells as well as by B cells is now quite firmly established. Much of the controversy surrounding T cell antigen recognition is concerned with the number and nature of recognition receptor sites on T cells, in particular the role of surface Ig on T cells. Many studies using cellular antigens, anti-θ-serum and the rosetting technique showed specific T-cell binding both in normal (291) and in immune animals (246,292-295). However, Schlesinger (292) and Wilson and Miller (294) were unable to find T-RFC in unimmunized animals nor could a number of other investigators find any θ +ve RFC in immune animals (296-299). Absence of T-RFC was observed in other species, e.g. chicken (300,301) and guinea pig (302). This variability, however, may be due in part to the specificity of the anti-θ-sera (303,304), although Elliott and Haskill (305) and Haskill et al (306) indicated that the main problem may be the relative instability of the T cell rosette, particularly with cells from unprimed mice. Other indirect and direct evidences confirmed the existence of antigen binding T cells. Thus, in a careful analysis of ABC in mouse spleen cell suspensions treated with anti-θ-serum, Roelants (253) clearly showed that about 25% of splenic ABC are θ-positive. In studies with (TG)-A-L, Hammerling and McDevitt (307) also showed that T cells bind antigen. Several studies have shown that the large number of ABC in the thymus, particularly fetal thymuses, cannot be accounted for by immigrant B cells (218,260,262,270,308).

The antigen binding specificity of cell surface-labelled Ig from activated T cells has been shown by Marchalonis and co-workers for a range of antigens including allogeneic cells (309), tumor cells (310) and protein antigens (311) and in all cases, it was concluded that the activity could not be accounted for by B cell contamination. Finally, a radioactive antigen suicide technique also convincingly showed that both normal T cells, antigen-activated helper T cells (312-314) and T cells mediating delayed-type hypersensitivity were capable antigen binding (314).

Immunization of animals and man results in a marked increase in the proportion of specific ABC (120,274,276,286, 315) whereas the induction of immunological tolerance has been varyingly reported to have no effect (247,316-318), to lead to a reduction in number (249,319-321) or a selective increase (247-249,322). Other workers found no thymus derived antigen binding cells in tolerant mice (248,322-324).

In summary, it is apparent that both B and T lymphocytes bind antigens by an immunologically specific process. In general there is an increase in the number of specific antigen binding cells with immunization, while tolerization diminishes this population of cells. The structure by which these cells recognize and bind antigen will be discussed in the following section.

2. Antigen Receptors

i. Nature of Ag Receptor

Since the response to antigen is characterized by the extreme specificity of the reaction, it is likely that a subpopulation of lymphocytes reacts with the appropriate antigen because of the presence of membrane associated recognition sites which are structurally complementary to the antigen (325-328). Also, as discussed previously, since both T and B cells carry surface Ig and receptors for antigen, it is not surprising that these antigen binding sites are considered to be intrinsic cell-bound antibody molecules or active fragments thereof. The binding of antigen by the cell through its reaction with this antibody receptor probably initiates the sequence of cellular and intercellular events leading to antibody formation and or cell-mediated immune reactions. There is ample evidence in the literature indicating the immunoglobulin nature of these receptors on the surface of lymphoid cells. Most studies utilized anti-sera to Ig or their components; their results will be discussed under three general headings:

A. Ability of Anti-Ig to Induce Transformation and Mitosis of Treated Lymphoid Cells

Rabbit peripheral lymphocytes can be stimulated in vitro to transform into blasts by incubation with homologous anti-serum directed against light and heavy chain allotypic determinants (329-331). These determinants were shown not to be passively absorbed by Sell and collaborators (145).

Heterologous antisera to whole rabbit serum (332,333), to specific Ig (334) or their subunits (335) are also mitogenic. Chicken spleen lymphocytes were shown to have receptors with μ chain specificity (336) as demonstrated by their capacity to undergo blastogenesis when incubated with rabbit anti-μ chain antisera. Blastogenesis occurred when human thoracic duct and peripheral small lymphocytes were cultured in vitro with anti-IgM and anti-IgG sera (337, 338). Daguillard and Richter (333) also showed that lymphocytes from humans gave a blastogenic response when stimulated with anti-Ig serum.

It has also been observed that immune rabbit lymphoid cells are incapable of transferring specific antibody-forming capacity if they are pre-incubated with antirabbit Ig serum prior to their injection into irradiated recipients. These cells also failed to undergo blastogenesis in response to stimulation with the specific antigen in vitro (333). These findings strongly suggest that the antigen and the anti-immunoglobulin antibody compete for the same site on the immunocyte surface and that the antigen binding site is an antibody.

B. Anti-Ig Inhibition of Antigen Binding Cells

(a) Inhibition of "B" Cell ABC

Pretreatment of ABC from several species, e.g. mouse (206,270,318,319,339-342), rabbit (118,343) and guinea pig (319) with anti-Ig of varying specificities has generally indicated that virtually all ABC are inhibited with polyvalent anti-Ig serum (251). In addition, IgM is shown to be

the predominant antigen receptor on B cells from unimmunized animals with the exception of the guinea pig, where most of the cells are inhibited by anti-γ_2 reagents (319). In immune animals, on the other hand, there is a clear shift to IgG-type receptors (118,341-343).

(b) Inhibition of T Cell ABC

Inhibition of rosette formation by anti-Ig was first shown by Biozzi et al (344,345), Zaalberg et al (346) and McConnell et al (347) and have shown complete inhibition. Biozzi et al (345) found that pretreatment of immune lymph node.cells from SRBC immunized guinea pigs with anti-guinea-pig γ-globulin antisera abolished their capacity to form rosettes in the presence of SRBC in vitro, implying that anti-globulin antibody could successfully compete with the antigen for the same site on the lymphocyte surface. McConnell et al (347) observed that rosette forming ability of SRBC immune mouse lymph node cells could be inhibited by prior treatment with anti-heavy chain antisera. This finding strongly suggests that the specific receptor on the immune lymphocyte is, in fact, an antibody immunoglobulin molecule or an active segment of the molecule. By using semipurified (cotton wool-filtered) preparation of T cells, Greaves (348) clearly showed that virtually all T rosette forming cells (RFC) could be inhibited by anti-Fab serum but not by anti-H-chain serum. However, Hogg and Greaves (349) found that some anti-μ chain sera cause up to 85% inhibition, while others gave only around 20% inhibition and that no other

anti-H-chain sera produced significant inhibition. It was soon found that those anti-μ chain with inhibitory activity had antibody to 'hinge' region determinants of the μ chain. These data implied that the IgM antigen receptor molecule on T cell is only partially exposed on the cell surface, the C-terminal of the H chain being inaccessible (350). A similar interpretation of T cell presentation of membrane bound 7s IgM was given by Marchalonis and Cone (164) utilizing radiolabelled surface Ig. That the antigen receptor on T cells contains Ig components was also shown by the ability of $F(ab^1)_2$ fractions of rabbit IgG anti-mouse K chain to inhibit specific antigen suicide (161) and the ability of anti-L-chain serum to inhibit suicide of activated T cells (314).

In summary, these findings and others (206,270,307,308, 351,352) clearly showed that inhibition of T rosette forming or antigen binding cell can be obtained with anti-Fab, anti-$F(ab^1)_2$, anti-K-chain and anti-μ-chain sera but not with any other anti-heavy chain sera even when primed T cells are used. Also, inhibition of antigen binding T cells is more readily achieved than antigen binding B cells (206,308). In further contrast to B cells, a phenotypic change in class of Ig receptor on T cells after priming is never observed (307). It was also shown that binding of antilymphocytic serum (206, 274,343) or anti-H-2 serum (307) does not inhibit antigen uptake, thus indicating that the presence of additional bound Ig does not sterically hinder antigen receptor binding.

Furthermore, the possibility that Ig is positioned extremely close to a non-Ig antigen receptor is virtually eliminated by the studies of Raff et al (353). Studies on inhibition of ABC by anti-Ig sera thus provided solid evidence in favor of the identity of antigen receptors and surface Ig on both B and T lymphocytes.

C. Anti-Ig Inhibition of B and T Cell Functions

Mitchison (326) first demonstrated the concept that cells carrying receptor antibody can react with antigen. In his experiment, he showed that anti-Fab or anti-IgG serum inhibited antigen stimulation of hapten-carrier primed cells. Similar results were obtained for a secondary response to keyhole limpet hemocyanin (354) and human serum albumin (333). It was also shown that pretreatment of unprimed cells with anti-K or anti-μ serum prior to their transfer to irradiated recipients markedly suppressed the subsequent antibody response to POL, Brucella and KLH (339).

Since these studies used either thymic independent antigens or haptens, it was concluded that the anti-Ig sera were inhibiting by binding to surface Ig on B lymphocytes. Similar conclusions were reached by Takahashi et al (296) and in these studies anti-θ serum was shown to be ineffective.

Inhibition of primary or secondary in vitro responses to SRBC with anti-Ig sera has also been reported by various groups (348,355-359) although in most of these studies only IgM responses were analyzed. Mond et al (359) was able to

restore the anti SRBC response with anti-θ treated spleen cells, therefore suggesting that the anti-Ig effect is on surface Ig bearing B cells.

In vivo experiments have also been undertaken to demonstrate the functional significance of surface bound immunoglobulin on lymphcytes.

The phenomenon of allotype suppression which results when fetal or neonatal animals are exposed to antiallotype antibody against one or both of the parental allotype (360, 361) serves as one example. Rabbits treated in this manner became extremely deficient in serum levels of the paternal allotype for many months or years. At the mature plasma cell level, it was shown that a decrease in number of plasma cells producing the suppressed allotype occurs (362). From in vitro lymphocyte stimulation studies with antiallotype sera, it was inferred that B lymphocytes bearing surface Ig of the suppressed allotype were also considerably reduced in number (363,364). This was confirmed recently by Harrison et al (365). Allotypic suppression was also found to occur in mice (272) and consists of two different types (366). In most strain combinations studied, only short-term suppression can be achieved (272) and is likely to be due to interaction of anti-Ig with surface Ig bearing cells. However, chronic suppression of SJL parental allotype in hybrid (B/c x SJL)F_1 mice can also be regularly achieved (367).

As with allotype suppression using anti-allotype sera injections of neonates with heterologous anti-Ig-class specific

sera suppresses the development of that Ig class and, in some instances, of other Ig classes as well. Thus purified goat anti-μ-chain antibodies when injected into embryonic chicken resulted in severe reduction in the synthesis of both IgM and IgG (368) as well as IgA (369). Similar studies in mice using anti-μ-chain treatment of neonatal germfree mice (370), conventional mice, or athymic nude mice (371) generated the following conclusions (370). Mice treated with anti-μ serum:

1/ had very few germinal centers in spleen and lymph node;

2/ had decreased numbers of mature plasma cells synthesizing IgM and IgG_1 in the spleen and virtual absence of IgA-synthesizing plasma cells in the gut;

3/ had greatly diminished numbers of B lymphocytes bearing membrane bound Ig of the IgM, IgG_1, IgG_2 and IgA classes in spleen;

4/ had reduced synthesis of IgM, IgG_2 and IgA by in vitro spleen cultures;

5/ had significant decreases in serum levels of IgM, IgG_1, IgG_2 and IgA;

6/ failed to make antibodies to ferritin after hyperimmunization;

7/ lacked natural antibodies to sheep erythrocytes.

Suppression of Ig formation by mouse spleen cells using heterologous anti-Ig sera has also been studied in an adoptive cell transfer system using allotype congenic mice

(372). Treatment of normal spleen cells with anti-μ antisera resulted in a tenfold or greater suppression of donor type IgG_1, IgG_{2a} and IgG_{2b} production after transfer of the cells to sublethally irradiated, allotypic congenic recipients. In these studies additional antigenic stimuli were not given, although from studies on the radiosensitivity of this allotype transfer (373) it is evident that B-cell proliferation is involved and that this system does not simply reflect secretion of Ig from already differentiated plasma cells. These data clearly point to the role of surface Ig on B cells as antigen receptors.

The role of surface Ig as the antigen receptor on T cells is much more difficult to demonstrate, particularly in view of the controversial nature of the very presence of surface Ig on T cells. Several approaches have however been developed to inhibit T cell function by anti-Ig in attempt to correlate the functional significance of surface Ig on T cells. T cell activity can be assessed in vitro by helper function in hapten-carrier antibody production, by stimulation of DNA synthesis with either antigens or mitogens and by the cytotoxic activity of activated T cells. In vivo functions include induction of delayed hypersensitivity and of allogeneic reactions. All of these systems have been studied for susceptibility to pre-treatment with anti-Ig sera, but there is a considerable lack of uniformity in the results obtained. Restrictions on the significance of positive results have been noted by Crone et al (201) but

on the other hand, inability to demonstrate an effect of anti-Ig serum does not necessarily imply that surface Ig is not the receptor site involved; the possibility that the particular Ig determinant recognized by the serum, if present on the cell, is not exposed or accessible to the serum cannot be excluded.

Both in vivo (374) and in vitro (375) activities of T cells were inhibited by anti-L-chain but not anti-H-chain sera. Pretreatment of human lymphocytes with rabbit anti-human L-chain antisera or its Fab monomer effectively depressed the response to PPD and to allogeneic cells (340,375-377) but not to PHA (375). In other studies, proliferative responses to antigen failed to be inhibited by anti-Ig sera (378). The in vitro responses of normal mouse spleen cells to TNP and erythrocyte antigen was inhibited by anti-L-chain sera and complement (379). By enhancing hapten responses in vitro using thymus derived, carrier-primed cells, it was shown that the inhibitory effects of anti-Ig is on the T cell. Inhibition of both hapten sensitive (B) and carrier-primed (T) cells with anti-Ig has also been observed in another study (380). However, these findings are contrary to those of others who failed to observe inhibition of either the generation of cytotoxic T cells in vitro or the effector stage of T-cell cytotoxicity to allogeneic antigens (381).

The in vivo effects of anti-Ig sera on graft versus host reactivity and delayed type hypersensitivity in mice and chickens are extremely variable. Three laboratories

reported a selective suppressive effect of anti-Ig on GVH reactivity, but not on hematopoietic stem cell colony activity (382-384). Suppression with Fab fragments of anti-Ig has been observed (385) although failure to suppress GVHR in mice has also been noted (386). Similarly, in chickens, although Crone et al (201) and Ivanyi et al (387) failed to suppress GVH reactivity with anti-L-chain, marked suppression was observed by Rouse and Warner (388). Studies with DTH have also produced conflicting results. In mice, Mason and Warner (374) reported a suppressive effect of anti-L-chain sera which Cooper and Ada (314) were unable to confirm. Using totally agammaglobulinemic bursectomized chicken, Theis and Thorbecke (389) demonstrated the in vivo suppressive effects of anti-L-chain antibody on DTH in this species.

In vivo or in vitro inhibition of T cell responsiveness by anti-Ig sera has thus been demonstrated in variety of systems, but with poor reproducibility, in terms of different antisera, and consistency with a given method. Nevertheless, in several cases, the inhibitory effect has been clearly shown to be due to the anti-Ig antibody and strongly indicates that the surface Ig on T cells is the antigen receptor site. In the in vivo studies, coating of T cells with anti-Ig may well lead to opsonization of the cell, thus removing it from suitable antigen contact, without requiring that the surface Ig actually be the antigen receptor. In in vitro studies, this cannot be duplicated, and, particularly in view of the timing requirement for anti-Ig treatment in relation to

antigen (376), it is most probable that the receptor for Ag is the membrane bound Ig. Furthermore, in the light of studies on the fluidity of membrane bound Ig (390) it is rather unlikely that steric inhibition by surface Ig -- anti-Ig complexes can be invokedas a cause of blocking a hypothetical non Ig antigen receptor.

From the preceding in vivo and in vitro observations, it is apparent that both B and T lymphocytes have cell surface receptors for antigen that are either in whole or in part composed of membrane bound Ig. It is generally accepted that the surface Ig on B cells is the sole antigen receptor while on T cells, it is suggested that the specificity of recognition is controlled primarily by membrane bound Ig and that binding of antigen to the cell can be mediated solely by this receptor. However, the activation of T cells requires an additional interaction of the antigen with another cell surface structure that is codedfor by a polymorphic H-2 linked gene (161).

3. Mechanism of Binding

Even at the end of the last century, Ehrlich (391) postulated that animals produce various types of cells with receptors which are complimentary to the injected or invading bacterial or cellular antigens. As a consequence of interaction between the antigens and the closely fitting "receptors" on the cell surface, the cells are stimulated for the regeneration of the homologous "receptors" which

later pass into the blood as antibodies. Jerne's natural selection theory (801) may be considered the first to revive Ehrlich's theory of antibody formation. Shortly thereafter, Burnet (4) modified the Ehrlich-Jerne's selective theory and introduced his clonal selection theory of acquired immunity. Burnet postulated that antigen sensitive cells are equipped with specific receptors through which antigen activation occurs thereby resulting in the development of a clone of antibody producing cells, all secreting antibodies specific for a given antigen. Jerne (392) and Mitchison (326) have logically concluded on the basis of the exquisite specificity of the immune system that only antibody recognizes antigen. Therefore immunoglobulin or immunoglobulin-like receptors for antigen on virgin lymphocytes is a logical choice. In summary, anti-Ig or its fragments have been shown to block the uptake of radioactively labelled antigens by normal lymphoid cells (251,339,340), to block the retention of immunocompetent cells by antigen-coated columns (393,394), to inhibit rosette formation (344, 347,350) and to inhibit both B (341,368,371,370,395) and T (374,375) cell functions. These findings strongly support the thesis that both B and T cells bind antigens through Ig molecules on their surface via a mechanism similar to that occurring in antigen-antibody reactions.

IV. CELL INTERACTION (C.I.)

1. Humoral Immunity

The functional compartmentalization of immunocompetent lymphocytes into T and B classes has been described previously. In recent years, it has been realized and now firmly established that these distinct lymphoid cell lines not only perform different roles in the generation of different forms of immunity but also interact synergistically with each other in the development of certain immune responses. The requirement of T and B cell interaction in eliciting antibody responses was first suggested by results obtained from studies in neonatally thymectomized mice some fifteen years ago (60,434-436). These studies demonstrated that neonatal thymectomy in mice prevented the normal development of cellular immunity exemplified by the homograft reaction as well as humoral response to such antigens as sheep erythrocytes (SRBC) and foreign serum proteins. The fact that implantation of a thymus graft restored, at least partially, the immune responsiveness of these animals (60, 64,434,437,438) and that bone marrow reconstituted, lethally irradiated, C57Bl mice regained full immunocompetence only if the thymus was present (439) indicated that the "T" cell component was needed for the full expression of immunocompetence. Several other observations indirectly suggested requirement of cell interactions in antibody responses. These include the "premium effect" described by Celada (440)

and the discovery by Gregory and Lajtha (441) that while the number of hemolytic foci in spleens of irradiated mice injected with syngeneic spleen cells and SRBC was linearly related to number of spleen cells transferred, the number of PFC increased dispoportionately. These results were consistent with the view that hemolytic foci development depends on one cell type whereas the production of a PFC requires an interaction of two cells neither of which is in excess. A similar effect was noted when peritoneal cells were cultured in vitro (442).

The first direct evidence for T and B cell interaction was provided by Claman and co-workers in studies of the humoral response to SRBC in mice (443-445). Using both the hemolytic plaque assay (446) and the hemolytic foci assay (447), these workers demonstrated that syngeneic thymus and marrow cell combinations were essential to achieve an adequate anti-SRBC response in lethally irradiated mice. Living syngeneic thymus cells were required since sonicated or irradiated mouse thymus cells or living heterologous thymus cells were incapble of transferring immunocompetence. Similar findings were reported by Davies and collaborators (27,69). Using chromosomally marked radiation chimeras, they were able to show, in addition to synergism between B and T cells, that thymus-derived lymphocytes responded proliferatively to antigenic stimulation (448) and that the antibody produced as a result of such stimuli is not synthesized by thymus-derived cells (19). Kennedy et al (449) also observed

that neither thymus cells nor bone marrow cells alone were capable of initiating hemolytic foci in recipient spleens, but the development of such foci occurred regularly when normal spleen or lymph node cells were used. In 1968, a series of elegant experiments by Miller, Mitchell and associates from the Walter and Eliza Hall Institute (5) provided considerable elucidation of the specific synergistic interactions between B and T cells. They have shown that neonatally thymectomized mice were restored in their capacity to respond to SRBC by transfusion of thymus cells or thoracic duct lymphocytes but that the anti-SRBC antibodies are synthesized by cells of the recipient (450,59). Employing anti H-2 sera (451) or the T_6 chromosomal marker (59) it was firmly established that the source of the PFC in this system was the bone marrow lymphocyte population. Shortly after initial studies demonstrating cellular interaction in the erythocyte system were reported, analogous observations have been made in the responses of mice to protein antigens (452-454) and to hapten-carrier conjugates in vivo (406,455-461). Co-operation between thymus and bone marrow cells has also been observed in rabbits (462-464). Improvements of tissue culture techniques by Mishell and Dutton (465) and by Marbrook (466) offered a useful approach to the study of specific cell interactions in vitro. However, co-operation in an in vitro response to SRBC could only be demonstrated if thymus and marrow cells were cultured separately for a few days in spleens of irradiated mice prior to culture in

vitro (467-469). There is one report by Doria et al (470) in which spleen cells from neonatally or adult thymectomized irradiated bone marrow chimeras could be restored by the addition of normal syngeneic thymus cells to respond to SRBC in vitro; and Schimpl and Wecker (471) have also reported that cortisone treated syngeneic thymocytes could restore the primary in vitro anti SRBC response of anti-θ serum treated mouse spleen cells. These studies demonstrated clearly that in vitro responses to SRBC mirrored the in vivo responses in its requirement for T and B cell interaction. In addition, the T cell component must be 'educated' with SRBC prior to mixing with the B cell which is the actual antibody synthesizing population.

In vitro studies have also provided convincing evidence that an adherent cell population, presumably macrophages, is required in addition to B and T cells for the production of humoral responses to some antigens (85, 472-479) but not to others (85,477-479).

The realization of the participation of two lymphoid cell types in the development of humoral responses generated an intense search for similar synergistic interactions in other facets of immunity such as immunologic memory, carrier effect, antigenic competition, tolerance and cell mediated immune reactions.

2. Immunologic Memory

The necessity of T cell co-operation for the production

of secondary immune responses has been demonstrated by Miller and his collaborators (187,480,481) in mice using a double transfer system, and was confirmed by others (482). Employing anti-θ serum and complement or other methods of specific elimination of T cells from immune spleens, other investigators have reported a loss of ability of such cells to transfer a secondary response to a thymus dependent antigen (429,483). Chan et al (469) also demonstrated in an in vitro system that educated thymus cells restored to normal the immune response of anti-θ sera and complement treated spleen cells.

A number of experiments in the early seventies first provided convincing evidence for the existence of memory in the B cell population, utilizing congenic mice, differing genetically only at the loci coding for immunoglobulin allotype, as donors. Jacobson et al (484) showed that all the IgG anti-SRBC PFC produced in an secondary adoptive transfer response were of the primed B cell allotype. Jehn and Karlin (485) showed that both thymocytes and bone marrow cells could adoptively transfer memory in the response to SRBC. It was also shown that elimination of B cells from an immune spleen resulted in definite reduction of secondary responses. Responsiveness could only be restored by immune B cells but not by normal spleen cells (197,380). Finally, specifically primed B cells but not unprimed B cells, when given with primed T cells were capable of enhancing the specific secondary response to the priming antigen in irradiated recipients (454).

3. Carrier Effect

The carrier effect was first described by Benacerraf et al (486) who first implicated the interaction of two cell types in the phenomenon. Essentially, he found that the secondary response to a hapten could only be obtained in the presence of both hapten and carrier primed spleen cells. This then was the first demonstration that different cells respond to the carrier and to the hapten; an interaction of these two cell types being necessary for an anti-hapten antibody response. Raff (405) subsequently showed that these carrier specific 'helper cells' are thymus derived as suggested by their sensitivity to anti-θ serum while the anti-hapten antibody forming cell precursors are not. The same co-operation phenomenon between carrier specific and hapten specific cells has been confirmed by many others (187,313,406,487-489) and shown also to occur in primary responses in vivo (460), in vitro (412,467,490,491) as well as in the secondary response in vitro (380).

Recently, a number of workers have shown that interaction between different subpopulations of T cells are necessary for generation of T helper cells. Utilizing a unique antigen with only two major antigenic determinants, performic acid oxidized ferrodoxin (OFD) derived from clostridium pasteurianum, Feldman et al (492) were able to demonstrate that the interaction of two T cell subpopulations is essential for the development of helper cells to OFD in vitro. In addition, using KLH as antigen, these investigators showed that helper

cell activity could only be obtained if defined populations of ALS and ATX treated spleen cells are combined. Hunter, Kappler and colleagues have also reported that both ALS and ATX diminish the helper responses to SRBC, but that both procedures are needed to abolish the helper response (493, 494). Moreover, Strobel has also shown that either ALS treatment or ATX markedly reduces the T-dependent antibody response to tobacco mosaic virus. Finally, Janeway (495) found that, when graded numbers of helper T cells were transferred adoptively to irradiated recipients given fixed number of B cells, an unexpectedly steep log dose-response curve of 1.67 was obtained indicating that there are two interacting cell types in the helper cell population. This phenomenon was termed the 'premium effect', since doubling the number of helper cells transferred leads to production of considerably more than twice as much antibody. Although successful in drastically reducing the helper activity, he was unable to abolish the premium effect by a variety of manipulations including anti-θ or anti-thymocyte serum plus complement treatment or adult thymectomy. The interpretation of these findings was that the premium effect is likely to be due to the co-operative interaction of two very similar types of mature T cells although the possibility that interaction of two different activities of a single T cell mediated by independent factors was not excluded.

4. Antigenic Competition

The phenomenon of antigenic competition, first recognized by Michaelis (1904) three quarters of a century ago, occurs when an animals is immunized with two antigens simultaneously or in close sequence resulting in a depressed antibody response to one or both antigens (476). The underlying mechanism has been the subject of much study but as yet remains unknown. Some investigators suggested the existence of a multipotential cell occurring with limited frequency (497,498) for which the antigens compete. This view was not accepted since it directly contradicts Burnets Clonal Selection theory. Kerbel and Eidinger suggested that, following antigen stimulation, a general shift in frequency of specific helper T cells occursthus diminishing the probability of interaction between specific B and T cells (499). Limitations of lymphoid space and nutrients have also been suggested as underlying cause of competition but again met with opposition. The most attractive explanation postulates the existence of soluble inhibitory factors (500-503) likely to be products of activated thymus derived cells. The competition phenomenon has also been described in systems of cell mediated immunity. Thus, large daily doses of soluble proteins for 8 to 10 days (504) or simultaneous injection of two antigens (505) led to inhibition of induction of delayed hypersensitivity to the challanging antigen. Similarly, the repeated injection of large doses of antigen led to a significant prolongation of homograft survival (506,507).

5. Cell-Mediated Immunity

Cell-mediated immune responses depend on T lymphocytes (508). T cells are essential for the development of cell-mediated immunity and cytotoxic effector cells are also T cells. B cells are thought to play no part in the induction of cell-mediated immunity (509-511). However, the recognition of T and B lymphocyte interactions in humoral immune response described previously prompted studies to determine whether analogous interactions existed in the ontogeny of cell-mediated immunity.

Globerson and Auerbach (512) found that in vitro GVH reactivity required presence of both thymus and bone marrow cells. Barchilon and Gershon (513) and Hilgard (514) reported observations of synergy between thymus and bone marrow cells in the production of GVH splenomegaly in X-irradiated F_1 recipients of parental donor cells. Synergism between T and B cells in the development of delayed hypersensitivity responses in mice have also been described (515). Despite these findings, it is generally believed that co-operative interactions between T cells are essential for production of cell-mediated immunity. Thus Cantor and Asofsky(516) reported synergy between thymus cells and another T cell present in spleen or lymph node in the generation of GVH reactivity. It was shown that one class of T cells consists of precursors of the effector cells and the other serves to amplify the activity of the former (511). Studies by Katz et al (517) in guinea pigs indicated that

the 'allogeneic effect' can enhance cellular as well as humoral immunological mechanisms. Similar results were obtained by McBride et al (518) using chickens. Several other investigators also demonstrated that synergism between subpopulations of thymus-derived cells in the development of different types of cell-mediated immunity (519-525) and that different reactivities may be involved in the recognition and effector phases of cell-mediated responses (526-530). Some evidence suggests that helper T cells in antibody formation and cytotoxic T cells may be members of different subsets of T cells (531). That accessory cells may facilitate the development of cell-mediated immunity has also been documented. Macrophages appear to be required for the proliferative response of sensitized T lymphocytes exposed to soluble antigens, apparently presenting antigen in an appropriate form (532). Removal of adherent cells greatly reduces the ability of spleen cells to generate cytotoxic lymphocytes in vitro (533-535). Adherent cells seemed to be essential for stimulation of proliferation in mixed lymphocyte cultures, although cell-free medium from cultures containing only adherent cells was able to substitute for these cells (536). Although it is necessary that the T and B cells be syngeneic for effective co-operation in humoral antibody responses (537), allogeneic macrophages may be able to serve as accessory cells in both antibody formation and in proliferative responses in mixed lymphocyte cultures (538). However, histocompatible macrophages have

been reported to be necessary for antigen-induced DNA synthesis by sensitized lymphocytes (539).

Synergism between T and B cells has also been described for immunologic tolerance (452,453,540-545) and will be discussed in more detail in a later section.

These studies therefore, clearly indicate that at least two functionally distinct but morphologically indistinquishable lymphoid cells interact synergistically in the production of an antibody response to most antigens in vivo and in vitro. One cell is thymus-derived and is stimulated to undergo mitosis (62,69,546,547) but is incapable of actual antibody synthesis (19,59,443,444,449-451); the other cell, of bone marrow origin, is the precursor of the antibody secreting cell and requires some form of co-operation from the thymus derived cell in order to function optimally (5,27,59,445,450). Interactions between cell populations also occur in the development of cell-mediated immunity; however, in this case, synergism is thought to be between two subpopulations of thymus-derived cells. Some evidence exists to include macrophages in the cellular interplay responsible for the generation of both humoral and cell-mediated immune responses. It is also obvious that cellular co-operation is necessary in manifestation of many immune phenomena.

6. Hypothesis of Mechanisms of Co-operation

The existence of collaboration between thymus-derived

and marrow-derived lymphocytes in antibody production as discussed previously, raised an immediate question regarding the nature and mechanism underlying these observations. A number of theories have been proposed but none escapes criticism entirely.

i. Focusing Theory

Perhaps the earliest proposal concerning the mechanism of T-B cell collaboration was the 'Antigen Focusing Theory' of Mitchison et al (548), postulated in an attempt to explain their findings in hapten-carrier systems. According to this theory, the function of the thymus-derived cells is to concentrate antigen on its surface via an immunoglobulin receptor (Ig X) (455), thereby presenting a large number of antigen molecules to antigen receptors on B cell surfaces. The idea is attractive in that T cells are known to comprise the bulk of the recirculating small lymphocytes (549) and are therefore particularly well suited for the role of binding antigen and concentrating them at sites where B cells reside. However, the theory also requires the T lymphocyte to be a much more efficient binder of antigen than B cells and only evidence to the contrary exists (319). Taylor and Iverson (550) proposed a modification of this model in which the IgX secreted by T cells acts via macrophages rather than directly with B cells in order to provide the appropriate trigger. Subsequently, Feldman and his associates provided evidence that T lymphocytes specifically bind antigen through

monomeric IgM, the Ag-monomeric IgM complex is then shed and attaches to macrophage surfaces where the antigen becomes properly orientated for presentation to and subsequent activation of B cells (551,552). These workers later extended their theory to include T cell dependent tolerance in B cells by showing that direct binding of such complexes to the receptors of B cells are tolerogenic (545). In addition, the phenomenon of antigenic competition was also explained on the basis of competition of various specific Ag-monomeric IgM complexes for available sites on the surfaces of macrophages; the unsuccessful and therefore 'free' complexes becoming a tolerogenic signal (553). The main objections to this version of the antigen focusing model is the difficulty in demonstrating the existence of 'IgT' and in proving that it is in fact a T cell product.

ii. Minimal Model

Essentially, the minimal model as proposed by Bretscher and Cohn (795) states that the B cell is only triggered when it receives a signal from its own receptor molecule and from the receptor molecule on, or provided by, the thymus-derived cell. This latter receptor molecule or 'carrier antibody', as it is termed, is equivalent to IgX in the previous model. The binding of antigen to B cell receptor, in the absence of carrier antibody results in tolerization of the B cell. This hypothesis provides a mechanism for the initiation of tolerance as well as the

discrimination of self versus non-self. To account for the responses of athymic animals to thymus independent antigens, the model postulates that these antigens require only very low concentration of carrier antibody to provide for the second signal, presumably due to the polymeric nature of these antigens. This argument is weakened, however, by the finding that T cell independent responses to polymeric flagellar proteins are obtained in vitro with only washed B cells (555,556).

An interesting variation of the minimal model has been suggested in which the T cell derived associative antibody or IgX is postulated to mediate B cell stimulation by activation of the third component of complement (C'3). In essence, the Fc portion of the IgX-Ag complex which is bound to the B cell antigen receptor, binds the C'3; the products of C'3 activation then stimulate the B cell to respond (557,558). Although Dukor and Hartmann (558) claim to have evidence that some T cell independent antigens have the unique property of activating C'3, it has been reported that mice depleted of C'3 can respond to T independent antigen such as pneumococcal polysaccharide type III antigen but not to T dependent antigens (557).

iii. Major Histocompatibility Complex Control of T-B Collaboration

Another alternative proposal to explain the mechanism of T-B co-operation comes from immunogenetic studies of

such collaboration and are supported by the apparent inability of the previous models to deal adequately with the genetic constraints of co-operation. In an immune response of mice to sheep red cells and other thymus dependent antigens, it was observed that syngeneic thymus and bone marrow derived cells co-operated much more efficiently than semi-allogeneic cells (451). This difference was accounted for by the disparity in MHC of donor animals (565). Subsequently, Katz and his colleagues (537) provided evidence suggesting that helper T cells and B cells derived from congenic mice with distinct histocompatibility regions failed to collaborate, or did so inefficiently unless certain critical genetic regions within the MHC were held in common, particularly the I region of the murine MHC. Janeway et al recently observed that similarity limited to the I-A subregion of the I region was sufficient to allow efficient collaboration between primed helper cells and B lymphocytes (559). A close parallel to these results was found in the studies of genetic constraints of macrophage-T lymphocyte interaction, in which sharing of certain alloantigens and of the Ir genes appears to be critical for T cell activation (539,560). These experiments suggest that products of the major histocompatibility complex, particularly of genes closely linked to immune response genes, play critical roles in the mediation of specific cellular collaboration.

iv. T Cell Mediator Model

This model requires the presence of a signal from the thymus-derived cell, in addition to the activation of B cells through its antigen receptor, as obligatory to the induction of an immune response. The T cell signal takes the form of soluble chemical mediator(s) which may or may not be antigen specific.

Various groups of workers have reported the presence of antigen specific factors (ASF) derived from T cells mediating co-operation. Kennedy et al (561) showed that supernatant obtained by gentle heating of peritoneal exudate lymphocytes is active in permitting B cells to develop antibody responses upon adoptive transfer to irradiated recipient mice. This factor is specific since it is only active if obtained from donors specifically immunized to the same antigen. Antigen specific migration inhibition factor (MIF) has also been reported by some workers (562). Spleen cells depleted of T cells were able to mount a primary in vitro anti-SRBC response in the presence of an antigen specific, non dialyzable factor, obtained from cultures of thymocytes and SRBC (563). Rokey et al (564) reported that a soluble, heat stable, 4S, probably proteinaceous factor obtained from normal bovine thymus, is capable of accelerating the production of specific hemolysin when administered to intact neonatal mice. Complexes of IgT and antigen were discussed by Feldman, Basten and colleagues (311,551,552,566). The presence of Ig mediators of co-operation has been confirmed

by Rieber and Riethmuller (567) and by Tada and colleagues (568). Active supernatants from antigen sensitized T cells have been shown to stimulate gamma-globulin synthesis and to elicit an antibody response from both sensitized and non-sensitized B cells (569). Other uncharacterized, soluble antigen specific factors have been reported by Yu and Gordon (570), by Gisler et al (571) and by Taussig (572,573). Also, it has been shown that the action of specific factors is dependent on an adherent cell (566) the exact nature of which is not known but is probably a macrophage (479,566) or dendritic cells known to retain antigen for long periods of time in vivo (35). Thus there is little doubt that antigen specific collaborative factors exist , but their exact molecular nature and their mechanistic action remain to be elucidated.

A large body of evidence also exists to implicate non-specific factors as critical to the mechanism of T-B co-operation. Most notable of these is perhaps the 'allogeneic effect' which occurs when lymphoid cells of different allotypes are exposed to each other and which have been shown to augment antibody production (574-580). Although the allogeneic effect may be due to a proliferative response on the part of the host T cell population, Katz and Benacerraf (581) suggested that it is more likely to be the result of some facilitative effect upon the precursors of antibody forming cells; probably via some type of non-specific, short-lived and fast acting soluble factors.

A number of workers have found that cell free supernatants obtained from cultures of allogeneic spleen cell (582-584) or thymus cell cultures (585-589) were able to reconstitute the immune capacity of lymphoid cell populations deprived of T lymphocytes. These and numerous other observations (468, 590, 592, 593) all gave support to the existence of nonspecific T cell factors capable of restoring and/or enhancing antibody production by B cells. Unfortunately, as with the specific factors, relatively little is known about the nature and mechanism of action of these nonspecific factors. Their action does not seem to depend on adherent cells, but affects B cell directly (594, 580); their production, however, may be macrophage dependent (595). While there is evidence to suggest that nonspecific factors promote B cell proliferation (580), others suggest that there is an increase in the number of B cells triggered. The actual mode of action is far from resolved.

At the present time, no single hypothesis proposed to explain the synergism between B and T cells can unequivocally account for all observed data. Nevertheless, what is plainly evident is the absolute requirement for the participation of the thymus-derived lymphocyte in many aspects of immunity.

V. REGULATION OF IMMUNE RESPONSES

It is well established that the extent of an immune response is limited both in time and magnitude; therefore, it is obvious that the initiation of an immune response

calls into play, either concomitantly or eventually, regulatory mechanisms which enforce these limitations. The immune response, being a complicated series of cellular events and interactions, readily lends itself to intervention at several different stages of development. Antibody, passively administered or actively produced, has been found to augment or inhibit the immune response and hence its involvment in regulation (433, 597-611). Bystryn et al (612) suggested that the regulation of antibody production is controlled via a dynamic equilibrium among circulating antibody, antigen and antigen-antibody complexes throughout the extravascular compartment. Alternately, regulation could occur locally at the site of stimulation and antibody synthesis (613, 614). Over the past few years, considerable data have been accumulated which strongly implicate subpopulation(s) of activated T cells or their products as regulators of the immune response. Since the nature of these regulatory mechanisms is of immediate relevance to the subject of this thesis, the topic will be reviewed in fair detail in the following sections.

1. Enhancement

i. Specific T Cell Influence

The helper influence of T cells on antibody production has been described previously. Briefly, T cell 'help' is needed for most IgG and some IgM antibody responses, and

possibly also for memory (615). Specific T cell help is illustrated most elegantly by the so called 'carrier effect' (187,406,548). Enhancement of antibody response as a result of specific priming or 'education' of T cells have also been reported (5,408,410-412,481,616,617), but in some cases, the augmentation is specific (187,412,468, 481,618), while in others, enhancement could be achieved with cells educated by a different antigen (408,619).

ii. Antibody Mediated

Considerable documentation of enhancement of anti-hapten responses by anti-carrier antibody exists (606,607,620-623) and this effect was found to be T cell dependent (624,625). Another interesting piece of information relating to enhancement of antibody responses by antibody is that of Playfair et al (616). They showed that the ability of educated thymus cells to co-operate with bone marrow derived cells in the response to SRBC in irradiated hosts was mediated by passively acquired antibody. In addition, their results showed that SRBC educated thymus cells enhanced the anti-SRBC response and that further augmentation was achieved when anti-SRBC antibody was given to the animal producing the educated T cells one day prior to harvesting. These workers postulated that activated T cells acquire a receptor for antibody or complexes with excess antibody, and thereby become competent to bind further antigen and co-operate, with specificity dictated by the antibody.

iii. Amplifier T Cell

Recently, there has emerged the concept of an amplifier T cell with a regulatory role in the immune response (410, 411,626). Thus, Baker et al (627) found that prior treatment of thymus bearing mice with various doses of anti-lymphocyte serum (ALS) all gave significant enhancement in their 19S PFC responses to SSS III; no such enhancement was found in nude mice. These workers reasoned that if the observed enhancement were due to the removal of suppressor T cells, then the same magnitude of response should be obtained in athymic nude mice as in neonatally or adult thymectomized mice or ALS treated normal mice when compared with normal intact mice. Since the response of nude mice was only one-hundredth of that produced by ALS treated thymus bearing littermates and only slightly greater than non ALS treated intact normals (627-630), it was proposed that nude mice lacked in addition to their normal complement of thymus-derived cells, a population of 'amplifier T cells'. The distinction of two subpopulations of T cells, one responsible for the basic helper cell function and another which regulates the expression of this function have been recently proposed by workers in our laboratory (411). It was postulated that the regulatory T cell may be an Fc+ subpopulation which has acquired complexes of cytophilic immunoglobulin and antigen. Baker et al (627) also contended that helper T cells (552) and regulatory T cells are independent of one another, and are mediated by different subpopulation of T cells. These subpopulations may differ with respect to tissue distribution,

susceptibility to ALS, minimal effective numbers and density and/or type of surface antigens (511,523).

iv. Non-specific T Cell Influence

Non-specific T cell mediated enhancement, best exemplified by the 'allogeneic effect' (631) has already been discussed previously. Furthemore, reports also exist in the literature demonstrating an enhancing effect of T cell mitogens on antibody production (632-635). On the other hand, little evidence could be found to indicate the enhancement of pure cell-mediated responses by T cells or their products. The most thoroughly studied example is probably that of Cantor and associates on the amplifying effects of one T cell subpopulation on another in the generation of graft-versus-host reactivity (511,516,636). Katz et al (517) and McBride et al (518) have also shown that the allogeneic effect resulted in the enhancement of cell-mediated responses in guinea pigs and chickens respectively. Finally, a recent study demonstrated that a Ly1+ subclass of peripheral T cells which is capable of providing helper activity during a primary antibody response (637) is also capable of amplifying the killer activity of another subclass of peripheral T cells (638).

2. T Cell Suppressive Influence

Based on the discussion above, it is evident that T cells have the capacity to augment both humoral and cellular

immunity in a specific as well as non-specific way. Recently, abundant evidence has also accumulated indicating that T cells are capable of regulating immune responses in a negative manner. This 'suppressor' activity may again be specific as well as non-specific. The responsible T cells have been coined 'suppressor cells' in an operational sense as it is not known whether they are a distinct class of T cells.

i. Tolerance Induction

In retrospect, the first direct suggestion that T cells have immunosuppressive effects came from the studies of Horiuchi and Waksman in 1968(639). They found that injection of antigen into the thymus of rats led to a significant and specific suppression of peripheral immunocompetence. Ha and Waksman (640) subsequently re-investigated these findings and found that thymocytes from BGG tolerant animals can, upon adoptive transfer to normal rats, specifically suppressed both cell-mediated and antibody responses to BGG. Shortly afterwards, Gershon introduced the term 'infectious tolerance' to describe the phenomenon of tolerant T cell populations exerting an inhibitory influence on normal cells, which in this case is an IgG response to SRBC (641). A similar 'infectious' nature of cells from putatively tolerant animals has been shown in certain states of acquired immunologic tolerance (642-653). In addition, specific suppressor T cell effects have been demonstrated with cells from non-

tolerant animals as well (545,640,654-662). This specific suppressor phenomenon is totally thymic dependent in both its inductive and effector phases, is operative in high (646,651) and low (647,663) zone tolerance, affects both cell-mediated (649,652,664,665) and humoral responses (543, 626,641-646,650,651,653), is activated by both soluble and particulate antigens and exhibits hapten and carrier specificity. Furthemore, T cell suppression of class specific responses has been demonstrated by Tada and his collaborators (666-669). These workers found that the homocytotrophic dinitrophenyl (DNP) antibody response of rats immunized with DNP-Ascaris could be increased by various manipulations which deplete T cells. This enhanced response could be rapidly abrogated by the inoculation of syngeneic carrier-primed but not BSA-DNP primed thymocytes. Similar findings were reported by Kishimoto and Ishizaka (670) in an in vitro system. Suppressor T cell activity has also been implicated as the mechanism for the in vitro induction of immunological tolerance (653,671). Others have observed suppressor activity in vitro from T cells of mice rendered unresponsive in vivo (646,672,673).

ii. Immune Responses to T-independent Antigen

Studies of thymus independent antigen induced immune responses have also contributed significantly to the concept of suppressor T cells. It has been known for some time that some antigens fail to demonstrate helper T cell activity in

normal animals (674,675). Examples of such antigens include pneumococcal polysaccharide type III (626,676-678), polyvinylpyrrolidone (PVP) (679,680) and bacterial lipopolysaccharide (endotoxin) (681,682). The immune response to these antigens can be augmented by a variety of ways which diminish T cells (677,680) but the enhancement is abolished by reconstitution of these T depleted mice with syngeneic thymocytes (677,681,682). Baker et al (627) extended their observations to congenitally athymic nude mice and found that depletion of their T cell complement had no enhancing effect in these animals. They attributed this lack to an absence of suppressor T cells in these animals.

iii. Antigenic Competition

Suppressor T cells or their products have also been implicated in some cases of antigenic competition (503,683, 684). Thus, a slight reduction of an animal's T cell pool, while having no or minimal effect on its capacity to mount an antibody response, will abrogate the competition phenomenon. Furthemore, normal cells added to an animal in which competition is occurring become inactivated and are subjected to the milieu effect (503,685,686). Nevertheless, conclusive demonstration of T cell derived suppressive factor(s) is still lacking, and in some cases, indirect participation of thymus dependent macrophages has been suggested (566,687).

iv. Autoimmunity

A number of investigators have recently presented evidence that a loss of suppressor cell function may result in outbreaks of autoimmune diseases in mice. Essentially they found that:

1/ T cells have a prophylactic role in prevention of autoimmune diseases (688,689), since

2/ depletion of T cells facilitates the production of autoantibodies and onset of autoimmune diseases in some strains of mice (689), chickens (690) and rats (691);

3/ thymus cells and thymus-derived splenic lymphocytes of young animals are less prone to develop autoimmune diseases than those of older animals and can in fact prevent or delay the onset of overt disease in older animals (689,692-696);

4/ Athymic nude mice are particularly prone to develop autoantibodies (697,698).

These findings are in agreement with the hypothesis put forward by Allison (699) that T cells exert specific feedback control on the synthesis of antibodies by B cells and that relaxation of this control, especially in aging humans and animals may be an important factor in the development of autoimmune diseases.

v. Tumor Immunology

The involvement of suppressor cells in the field of

tumor immunology is also becoming more evident. Splenectomy has been commonly noted to increase tumor immunity (700-702) and immune responses to tumors have been shown to be modulated by suppressor T cells (703-705). Fujimoto et al (706,707) have also recently demonstrated the presence of immunosuppressor T cells in the thymus and spleens of tumor bearing hosts exerting a specific negative effect on the immune response to homologous tumors. Furthemore, it was shown that in vivo treatment with anti-mouse thymocyte serum (ATS) or in vitro treatment of lymphoid cells of these tumor bearing animals with anti-theta antiserum and complement abolished the suppressor activity.

Using a technique which permits the study of terminal differentiation of B cells into Ig synthesizing and secreting cells, Waldmann and his associates analyzed the physiological and pathological factors regulating immunoglobulin synthesis and the role of suppressor T cells in this process (708-710). They essentially found that peripheral blood B cells from patients with common variable immunodeficiency thymoma and hypogammaglobulinemia failed to synthesize significant amounts of any Ig when cultured with pokeweed mitogen while normal peripheral B cells do. Indeed in many of the above mentioned cases, co-culture experiments showed that the suppressor T cells inhibited the stimulation by pokeweed mitogen of normal B cells to synthesize Ig. These observations suggested that the defect in these patients is caused or perpetuated by an abnormality of regulatory T cells which acts to suppress B cell maturation and antibody production.

vi. Genetically Determined Immunologic Unresponsiveness

A single gene (genes) closely linked to those which determine histocompatibility has (have) been shown in a number of species to determine the capacity to mount an immune response to certain well-defined antigens (711). In at least one such case, the basis for the inability of non-responder animals to generate an immune response to the antigen in question has been shown to be due to the relative ease with which T cells in these animals become tolerant to these antigens (712). This type of tolerance has again been shown to be modulated by suppressor T cells (713-716).

vii. Cell-mediated Immunity

Documentation of T cell suppressive influences in various aspects of cell-mediated immunity is also plentiful. Thus, suppressor T cells have been implicated in the regulation of proliferative response of other T subpopulations (717-719) in mixed lymphocyte reactions, in the regulation of the activities of cytotoxic lymphocytes and of graft-versus-host responses (720-725), in regulating the development and expression of delayed hypersensitivity responses (664,726, 727) and contact sensitivity to DNFB (728). Finally, it has been observed that subpopulations of Con A activated T cells release potent suppressive factors which apparently act by blocking macrophages (725,729-735).

In summary, it has been shown that the effects of suppressor T cells are antigen specific in some immune phenomena (641,642,644,664,674,715,726,727,736), while in others, although the suppressor T cells are activated by specific antigens, their suppressive effects are non-specific (366,647,674,683,720,737). Soluble factors released by suppressor T cells mediate the suppression of some immune responses (657,658,674,683,727,733,736) whereas suppression of other responses may require direct cell-to-cell contact (366,647,674,720,725,737). Suppressor T cells or their products may act directly on potentially responsive T or B cells, or indirectly on macrophages by mechanisms which are still undefined (366,647,657,658,664,674,683,715, 720,726,727,736,737). The numerous and diverse situations in which suppressor T cell activity has been documented strongly suggests that these cells play a very important role in immunoregulation. Although few in number, there is strong evidence now that suppressor cells may be a distinct population expressing Ly 2, 3 alloantigen (720,867,868) or I-J determinants (869). Nevertheless, based on the present existing information, the precise mechanism by which suppressor cells and/or their products operate is equally controversial and should provide an extremely challenging objective to researchers in this field.

VI. IMMUNOLOGIC TOLERANCE

Immunological tolerance is defined as a specific refractory state to an antigen which results from previous contact with the same antigen. A complexity of cellular events similar to that involved in the immune response is also involved in the induction of immunological unresponsiveness.

1. Sites of Immunological Unresponsiveness

One of the major issues has been to define the site of tolerance induction in a given experimental situation, that is whether T cells, B cells or both have been rendered unresponsive. It is now well established that both thymus-derived and bone marrow derived cells can be specifically tolerized.

That the thymus is involved in immunological unresponsiveness was first suggested by the prolongation of the unresponsive state in mice after thymectomy (738,739). More recently, bursectomy of chickens has been reported to prolong the unresponsive state to BSA (740). The first direct evidence that thymus-derived cells were affected during the induction of immunological unresponsiveness was documented by Isakovic et al (741) who demonstrated that thymus grafts from BGG tolerant rats transfered the specific tolerance to thymectomized irradiated recipients. Taylor observed unresponsiveness to BSA in thymus cells but not in bone marrow cells (452,540). Similarly, Many and Schwartz

(742) reported that cells from thymus but not bone marrow to be unresponsive to SRBC 48 hours after injection of SRBC and cyclophosphamide. Miller and Mitchell (743) were also able to induce tolerance to SRBC in mice with cyclophosphamide; in this case only lymphocytes in the thoracic duct lymph and spleen were tolerant. Paul et al (461) demonstrated that carrier specific T cells could be rendered specifically tolerant thereby abolishing their helper function in secondary anti-hapten antibody responses.

Playfair (541), using high doses of SRBC and cyclophosphamide to induce tolerance in mice, found that B cells were transiently tolerant. Talal et al (744) also observed a hyporesponsive state in bone marrow cells of mice treated with multiple injections of SRBC and cyclophosphamide while Gershon and Kondo (543) tolerized B cells by repeated injections of SRBC alone. More recently, B cell tolerance as reflected by hapten specific tolerance has been reported (745-748). Using the T independent antigen, pneumococcal polysaccharide, Howard (323) also demonstrated B cell tolerance. Perhaps the most clear cut demonstration that both T and B cells are sites of tolerance induction is contained in experiments performed by Chiller, Weigle and their associates (453,542,749-751). In an adoptive transfer system, these investigators showed that although both thymus and bone marrow cells could be rendered tolerant to HGG, critical differences exist for the two populations in terms of kinetics of tolerance induction and effective dosage of

tolerogen. Thus it was observed that the unresponsive state in thymus cells could be rapidly induced (2 days) with relatively small amounts of tolerogen (100 μg or less) and were of relative long duration. On the other hand, the unresponsive state in bone marrow cells was of short duration (49 days), slowly induced and required much larger doses of tolerogen (2.5 mg). In addition, these results made it obvious that tolerance in either T or B cell population will suffice to render the whole animal tolerant in cases of T dependent antigens. Unresponsiveness and hyporesponsive states to a number of antigens with diversified properties have been reported. The nature of the antigen plays a major role in the degree and duration of the unresponsive state produced. Specific immunological tolerance has been studied with purified serum proteins, viruses, bacteria and their products, heterologous erythrocytes, haptens and synthetic polypeptides. Only those cases which are of immediate relevance to this thesis and to the development of major proposed mechanisms of tolerance will be discussed in some detail.

2. Tolerance to Selected Antigens

i. Heterologous Serum Proteins

Heterologous serum proteins readily lend themselves to investigation of a number of aspects of immune tolerance. This is simply due to the ease of obtaining large amounts

of highly purifed protein, to their wide tissue distribution (35) and to their slow rate of removal from extracellular fluids. When free of aggregates these substances are extremely tolerogenic. One such protein, human gammaglobulin (HGG), had been employed in the studies of Chiller and associates as previously described. Mitchison (752,753) using bovine serum albumin (BSA) observed two zones of specific tolerance, a 'low zone' tolerance induced by small doses of antigen and a 'high zone' tolerance induced with large doses of antigen. Intermediate dosages resulted in induction of immunity (754). Subsequently, these investigators concluded that low zone tolerance affected T cells and high zone tolerance involved B cells. Rajewsky (755) also note the relative difficulty in rendering B cells tolerant and showed additionally that only those B cells whose progeny made high affinity antibodies were affected in successful cases (756). Miller et al (187) using fowl gammaglobulin (FGG) failed to produce B cell tolerance but readily obtained T cell tolerance. Similarly, Feldman and Nossal (552) showed that T cells were easily made unresponsive to FGG with 1 μg/ml of antigen while B cells were unaffected by 100 times this dose. Nevertheless, B cell tolerogenesis has been conveniently studied by complexing haptens onto autologous proteins (746, 757) or immunogenic carriers such as deaggregated heterologous globulin (758-760). Therefore, it seems fairly well established that soluble heterologous proteins can tolerize B cells, but at a higher dosage than is needed to tolerize

T cells.

A number of alternatives exist to account for tolerogenesis of T and B cells by soluble heterologous proteins. Receptor blockade by tolerogen was noted as one possibility, particularly in an vitro system (761), but a number of considerations argue against it being operative in intact animals. For example, the presence of a lag period of one day or more in tolerance induction would be inconsistent with the extremely rapid equilibration of antigen between extra and intravascular fluid compartments as well as with the equilibrium binding of antigen with receptor at 37°C. Simple blockade of receptors should be rapidly reversible upon adoptive transfer (747) probably due to metabolic turnover of receptors (762) but tolerance is stable upon adoptive transfer to tolerogen free environment. B cells coated with soluble deaggregated fowl gammaglobulin (763) can be triggered into antibody production upon exposure to mitogens such as bacterial endotoxin or polymerized flagellin. Finally, when B cells in athymic nude mice are rendered unresponsive, they appear to go through a stage where they can form antibody before becoming tolerant (764). An alternate explanation is that of clonal deletion as a result of contact with antigen (554,763-765). A third mechanism involves mediation by specific suppressor T cells (766). In this hypothesis, it was assumed that suppressor cell precursors have a short functional life span beyound which they can no longer differentiate into suppressor

cells. In addition, both helper or effector and suppressor cells presumably originate from some common precursor in the thymus. In the absence of antigenic stimulation, the immature thymic precursors eventually mature to form helper or effector cells which could be subsequently stimulated to produce an immune response. Antigen contact of these immature precursors at the correct stage of their development, prior to their becoming helper or effector cells, drives them to differentiate into specific suppressor cells. This would also have the effect of depleting the common precursor cell pool and thereby the source of recruitment of helper or effector cells. The end result would be a specific and observable unresponsiveness to that particular antigen. This explanation would account for high zone tolerance, while low zone tolerance could be explained by the assumption of a lower threshold of responsiveness of the immature thymic precursors so that subimmunizing antigen doses would preferentially stimulate the immature cells to differentiate into specific suppressors.

ii. Antigens with Multiple Repeating Determinants

The use of antigens with multiple repeating determinants, of which pneumococcal polysaccharides (323), bacterial endotoxins (767) and polymerized flagellin (552) are typical examples, as agents in the induction of unreactivity had been well documented. Thus, immunological unresponsiveness can be induced in adult mice after injection of moderate

amounts of purified pneumococcal polysaccharides as first observed by Felton (768) and subsequently by Howard and co-workers (323). Injections of minute amounts of antigen resulted in an apparent unresponsive state characterized by the absence of circulating antibody but the presence of antibody producing cells remains (248). This pseudo-unresponsive state probably resulted from a treadmill neutralization of the antibody by the persisting antigen (769). Higher doses, however, resulted in central inhibition and the disappearance of antibody producing cells. In contrast, a high dose of the polyvalent antigen, levan, causes tolerance, apparently without prior immunization or treadmill neutralization (770,771).

An unresponsive state to E. coli endotoxin had been observed in mice following injection of detoxified endotoxin into adult animals (767). Complete tolerance to the monomeric subunit (flagellin) of the flagella of Salmonella adelaide had been induced in neonatal rats (772) while adult animals became unresponsive to flagellin following pretreatment with acetoacetylated flagellin (773). Nossal and Ada (35) also showed that polymerized flagellin (POL) in high doses was a potent tolerogen both in vivo as well as in vitro. Diener and Paetkau (256) using H^3-labelled POL found that immunogenic concentrations of antigens caused patch and cap formation which persisted for several hours in the continued presence of antigen. Higher tolerogenic concentrations, however, initially caused capping but this

was followed by a random distribution of the receptors. These and other observations (774,775) prompted the speculation that tolerogenesis to a polymeric antigen depended upon the quantity of antigen associated with the surfaces of immunocompetent cells; when the number of antigen recognition sites cross-linked by repeating antigenic determinants is above a critical threshold concentration, tolerance results. This hypothesis was also supported by the observation that the 2-4-dinitrophenyl hapten coupled at a high substitution ratio to a co-polymer of D-glutamic acid and D-lysine resulted in a potent hapten specific B cell tolerogen (747,776).

iii. SRBC

Tolerance to SRBC has been obtained both in neonatal and adult animals by a prolonged injection schedule with massive doses of antigen (777-779). More generally, suppression has been achieved by treatment with antigen in combination with cytotoxic drugs such as cyclophosphamide (448,549,641,742,743,780-784). Solubilized SRBC fractions have also been used in induction of tolerance to SRBC (417, 785-787). Depending on the schedule of injections of antigen, the number and source of lymphoid cells used in reconstitution experiments and the time at which the immune status of the experimental animals were assessed, tolerance to SRBC has been variously attributed to a loss or inactivation of T cells or B cells or combinations of both (641).

In addition, the possibility of participation of suppressor cells have also been proposed (641,729,788). Recently, Auerbach and his collaborators, using a solubilized and non-antigenic fraction of SRBC, was able to induce long lasting and specific tolerance in adult mice. However, spleen cells from such tolerant animals were responsive to SRBC challenge in normal irradiated hosts and when cultured with the antigen in vitro (785). The unresponsiveness in this case was found to be mediated by soluble factors, released from lysed SRBC, which were distinct from the major antigenic determinants normally recognized as foreign as they were totally non-immunogenic. These investigators suggested that the inhibitory factors or 'tolerogenic determinants' may be capable of eliciting specific blocking activities (including antibody or antigen-antibody complexes) which prevent the stimulation of specific immunocompetent cells via steric hindrance type of mechanism. The essential requirement of the hypothesis is the close approximation between the tolerogenic determinants and the major antigenic determinants of the SRBC. Such a mechanism of steric tolerance could explain the high degree of efficiency of self recognition despite the continuous emergence of new antigens during adult life, since new antigens would not be recognized as foreign if the tolerogenic determinants did not change. Furthermore, autoimmunity as seen in the framework of steric tolerance, results when responses to the tolerogenic

determinants diminish. Also, immune surveillance would break down when the neoplastic cells retain the tolerogenic determinant of the cell from which they arise.

3. Antibody Mediated Suppression

It has long been known that passive antibody can hamper an active immune response. The necessity and relevance of an understanding of the role of antibody and/or antigen-antibody complexes became clear when serum blocking factors capable of interfering with cytotoxic killing of tumor cells were discovered (789,790).

Two broad categories of antibody mediated suppression may be distinquished. First, there is the conceptually simple peripheral suppression (597) where the high concentration of serum antibody competes successfully against surface receptor antibodies for the limited amount of antigen. This does not involve any changes in the lymphoid cells. A second more interesting mechanism involves central suppression which occurs when lymphocytes are exposed to a mixture of antigen and antibody with antigen being slightly in excess (598,791). While documentation of such an effect on B cells is most complete, it has also been shown that a mixture of FGG and anti-FGG can abolish the helper function of FGG primed T cells (552). In effect, what happens is that in the presence of antigen excess soluble complexes of antigen and antibody forms with significant number of antigenic determinants unoccupied by antibody. Such an

antigenic configuration closely resembles that of a multivalent antigen with repeating determinants and is therefore tolerogenic as previously discussed. Such a mechanism of central suppression was not confined to any one group of antigens, but was found to underly specific tolerance to all the variants of the flagellin molecule, for serum protein antigens and for soluble sheep erythrocyte antigen (774). It is an attractive proposal as it provides an effective feedback loop in immune regulation and effectively explains those forms of tolerance induced by repeated injections of small amounts of antigen (792), where some antibody formation usually precedes the tolerant state. In addition, the concept of self tolerance would also be satisfactorily explained by such a mechanism. Soluble complexes have been suggested to cause effector cell blockade in cases of tumor or allograft enhancement (790,793,794). In Burkett's lymphoma, the level of membrane reactive (anti-MA) antibodies tends to stay at a plateau level in patients in remission. When a sudden fall in anti-MA antibody level occurs, a clinical relapse frequently follows within a short time. It is likely that the fall in antibody levels is due to complexing with antigens, the resulting complexes exerting an inhibitory effect on the immune response which held the tumor cells in check (801). The studies of Nossal and co-workers, involving micro-manipulations of single antibody producing cells, also showed that hyporeactivity can be induced in B cells via soluble complexes, even at the later stages of differentiation

such as during antibody production (760).

4. Mechanism of Tolerance Induction

Based on the above discussion, it is evident that no one single mechanism could satisfactorily account for various types of tolerant states. It is extremely likely that several pathways exist depending on the particular experimental conditions. Furthemore, it is quite conceivable that more than one mechanism may act in concert to produce the final observed state of immunological unresponsiveness. Nevertheless, the existing data on mechanisms of tolerance induction can be roughly grouped under two major headings, central unresponsiveness versus peripheral inhibition.

The concept of central unresponsiveness originates as an offshoot of Burnet's Clonal Selection Theory (4) and postulates that immunocompetent cells of a given specificity are eliminated on contact with antigen during a critical phase of their maturation before they become capable of activation by antigen. This theory allows discrimination between self and non-self since self antigens are continuously present and will abort any self-reactive immunocompetent cell it contacts before the latter matures into an antigen reactive cell.

In contrast to central unresponsiveness, other states of experimentally induced tolerance exist in which mature competent cells are present but cannot be stimulated to produce a detectable response. In these cases, the

immunocompetent cells may be suppressed by appropriate modulation of regulatory mechanisms of the lymphoid system resulting in peripheral inhibition of the immune response. One such hypothesis proposes that contacts with antigen can either be stimulatory or inhibitory and finds support from the two signal theory of immunity-tolerance signal discrimination (554,765,763). According to this theory, two signals, one from union of antigen and surface receptor and the other from activated T cells or macrophages, are required for activation. In the absence of the latter signal, the antigen sensitive cell is turned off and tolerance ensues. Such a mechanism may possibly occur in models where autoradiography showed a reduction in the number of immunocytes capable of binding the tolerogen (321). Another example is the tolerant state induced by either pneumococcal polysaccharides or the lipopolysaccharides of Gram negative bacteria. It was shown that the spleen cells from tolerant mice responded normally to an immunogenic challenge with homologous antigen when transferred to irradiated normal recipients (322,323). Additional features of peripheral inhibitory states include the presence of specific antigen binding lymphocytes in the tolerant animals and the occurrence of a transient state of antibody production prior to establishment of the tolerant state. Tolerance to tumors (609,796-798) and possibly to transplantation antigens (799, 800) on the other hand, probably involves a different type of peripheral inhibition. Both are

characterized by immunity rather than an unresponsive state; antibody probably plays a critical role in inhibiting the rejection of foreign tissues by cytotoxic T cells. It is also possible that peripheral inhibition is mediated by suppressive T cells. Such suppressor cells apparently exist as part of the network of the regulatory mechanisms of the immune system and have, indeed, been implicated in a number of immunological unresponsive states (discussed under section on suppression).

It would seem pertinent at this point, to review briefly, some of the basic observations made in our laboratory which provided the framework for this thesis.

Firstly, an increase of Ig carrying lymphocytes was consistently observed in the spleens of Balb/c mice 6 hours following antigenic stimulation. This increase amounts to 20-25% of preimmunization levels and was elicited by soluble protein antigens in the presence of FCA or particulate antigens without FCA (1,2). Subsequently it was conclusively shown that such an increase in Ig positive cells is due to the uptake of cytophilic IgG by a subpopulation of T cells in the spleen (2,413). This conclusion was supported by evidence from several different experiments: (a) it was observed that there is a decrease of theta-carrying cells at 6 hours which is equal in magnitude to the increase of Ig-carrying cells present at this time; (b) following antitheta antiserum plus complement treatment, normal spleen cells lost the ability to exhibit an increase in Ig-carrying cells when incubated with serum containing potent cytophilic

activity, while anti Ig antiserum plus complement treatments did not affect this ability; (c) only thymus cells but not bone marrow cells incubated with serum containing potent cytophilic activity were able to express an increase in Ig positive cells; (d) finally spleen cells from sublethally irradiated and thymus but not bone marrow cells reconstituted hosts showed an increase in Ig positive cells 6 hours following antigen challenge.

Having established that such an increase was due to the uptake of cytophilic Ig by T cells, it was next demonstrated that this cytophilic Ig represents complexes of antigen and mouse Ig (3,413).

Finally, further investigations revealed that the formation of such cytophilic Ig-antigen complexes is mediated by a soluble factor (tentatively called Ig antigen complexing factor or IACF), which is produced by T cells (413,414).This factor could either be recovered from the 4s fraction of serum collected from animals challenged 6 hours previously with FCA or from supernates of thymocytes cultured briefly with a variety of antigens such as mycobacteria, heterologous erythrocytes, allogeneic cells, LPS or polyadenylic:polyuridylic acids. These factors are capable of inducing complexes which are cytophilic for T cells only in the presence, simultaneously, of immunoglobulin and antigen.

These data raised a number of interesting questions. What, for example, is the mechanism of uptake of these

cytophilic complexes by T cells? What is the nature of IACF? What is the origin and nature of Ig in these complexes? Perhaps the one most pressing question is that of the functional role of these Ig carrying 6 hour T cells in immunity. Accordingly, two different experimental approaches were undertaken to study this point. The first approach utilizes an adoptive transfer system to test directly the role of 6 hour T cells in antibody production. The second approach consists of abrogating the antibody response to an antigen and observing what effects such a manipulation would have upon 6 hour changes, soluble factors and cytophilic complexes. The work presented in this thesis represents attempts along the second approach, and it is hoped that the two approaches will compliment each other in terms of defining the role of the six hour T cell in immunity.

MATERIALS AND METHODS

MATERIALS AND METHODS

I. Mice

Inbred BALB/C mice 6-10 weeks old were obtained from Canadian Breeding (Montreal). C3H, AKR and C57B1 mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

II. Rabbits

Outbred white rabbits, weighing about 5 lbs, obtained from Canadian Breeding Laboratories, St. Constant, Quebec, were used for the preparation of rabbit anti-BSA and antimouse Ig sera.

III. Horse and Sheep Erythrocyte Hemolysate Supernatant

Horse and sheep erythrocyte hemolysate supernatant abbreviated HHS and SHS respectively, were prepared according to the method of Anderson et al (417). Fresh horse or sheep erthrocytes (HRBC or SRBC)(National Biological Laboratory Supplies, Winnipeg, Manitoba) were washed with physiological (0.15M) saline at least three times or until the supernates were clear. The red blood cells were spun down after the last washing and subjected to hypotonic lysis in distilled, demineralized water at a volume ration of 3:1 distilled water to packed erythrocytes. The lysed red blood cell solution was then spun at 1000G for 10 minutes at $4^{o}C$ to remove leukocytes and the supernatant centrifuged at 34000G for a minimum of 200 minutes at $4^{o}C$ using a Ti 50 rotor in an L2 preparative ultracentrifuge (Beckman Corp.,Toronto,Ontario). The upper third of the

resultant supernatant was removed and readjusted to 0.9% salinity with sodium chloride and then filter sterilized through 0.45μ Millipore filters under pressure. The sterilized preparation was then alloted into 20ml aliquots in sterile bottles and stored frozen at -20°C until use. Once defrosted for use, the material was never refrozen but kept at 4°C still under sterile conditions and used up within a week, or otherwise discarded.

IV. Antigens

1. Mouse IgG and IgF Myeloma Proteins

Mouse IgG (IgG2a) and IgF(IgG1) myeloma proteins were isolated from ascitic fluids of plasmacytoma bearing Balb/c mice by diethylaminoethyl (DEAE) anion exchange columns equilibrated in 0.005 M phosphate buffer, pH. 7.5. The proteins were eluted by stepwise increase of buffer molarity from 0.005 M to 0.01 M and finally to 0.033 M as recommended by Potter (418). The eluates were collected in 5-7 ml fractions by an automatic fraction collector and the protein content of each tube determined by measuring the optical density at 280nm with a Zeiss M4QIII Spectrophotometer (Carl Zeiss, Oberkochen, Wuerttemberg). All tubes of a given peak were pooled and then dialyzed in cellulose tubing against 0.15 M borate saline buffer, pH 8.0. The equilibrated material was then concentrated by ultrafiltration and examined by immunoelectrophoresis using a polyvalent

rabbit anti whole mouse serum. The presence of IgG myeloma protein was found in the 0.005 M and 0.01 M fractions, while the IgF myeloma protein was found in the 0.01 M and 0.033 M fractions. To eliminate trace contamination of transferrin, the myeloma proteins were further purified by gel filtration on Sephadex G200 column equilibrated in 0.15 M borate saline buffer, pH 8.0. Examination by immunoelectrophoresis and Ouchterlony indicated the presence of a single Ig class in the first eluted peak.

2. Horse Spleen Ferritin

Horse spleen ferritin (Fe) 2x crystallized, cadmium free, was purchased from Nutritional Brochemical Corp., Cleveland, Ohio.

3. Bovine Serum Albumin (BSA) and Chicken Egg Albumin (EA)

Both proteins were obtained from Pentex, Kankakee, Illionois.

4. Human Fibrinogen

Human fibrinogen (FIB) was bought from Connaught Medical Reseach Laboratories, Toronto, Canada.

5. Chicken Erythrocytes

Chicken blood was collected fresh in 0.1 M citrate buffer, pH 4.8, from DunnRite Co., Winnipeg, Canada and the red blood cells (CRBC) washed three times in 0.15 M saline before use.

6. Horse and Sheep Erythrocytes

Horse and sheep blood were purchased from National Biological Laboratories, Winnipeg, Manitoba. The red cells were washed three times in 0.15 M saline before use.

V. Adjuvants

Freund's Complete Adjuvant (FCA) was obtained from Difco Laboratories, Detroit, Michigan.

BCG Vaccine was purchased from Connaught Medical Research Laboratories, Toronto, Canada.

VI. Rabbit Immunization and Collection of Antisera

Rabbits were immunized by intramuscular injection with a mixture containing 1mg protein and 0.5ml each of 0.15 M saline and FCA. The animals were injected once a week for 5-6 weeks and then test bled via an ear vein one week after the last immunization. The antibody activity of each antiserum was checked by immunoelectrophoresis and upon confirmation of the proper antibody activity and titre, the rabbits were bled by cardiac puncture once a week for 4-6 weeks. Following a rest period, a booster shot of antigen was given and the animals bled a week afterwards. At each bleeding, 50ml of blood was collected and allowed to stand at room temperature (R.T.) for a few hours before the serum was separated from the clot by centrifugation at 3000 rpm for 15 minutes. The sera were then stored at $-20^{\circ}C$.

VII. Mouse Immunization and Collection of Sera

Mice were usually immunized with the appropriate antigen in 0.2 ml volume intraperitoneally (i.p.). For soluble protein antigens such as FIB, EA and Fe, 250 ug of protein in 0.1 ml of 0.15 M saline emulsified with 0.1 ml of FCA was used. With particulate antigens such as CRBC, SRBC and HRBC, 2×10^8 red blood cells were administered in 0.2 ml of 0.15 M saline. When allogeneic spleen cells were used as antigen, 15×10^6 6x frozen and thawed (6x) spleen cells were injected in 0.2 ml of 0.15 M saline. In the case of BCG, 0.1 mg in 0.2 ml of 0.15 M saline was used.

Blood was obtained by heart puncture and allowed to stand at R.T. for a few hours before centrifugation at 3000 rpm for 5 minutes. The serum was separated from the clot and stored at -20°C until use or at 4°C and used within a couple of days.

VIII. Ion Exchange Chromatography by DEAE Cellulose

The DEAE cellulose ion exchange column was packed according to the method of King (419). Dry DEAE cellulose (Carl Schleicher and Schuell Co., Keene, N.H. 0.89 meq/gm dry wt.) was washed by suspending it in 0.5 N NaOH and 0.5N NaCl with continuous stirring. The suspension was allowed to settle for 30 minutes, and the cloudy supernatant was decanted. The cellulose was resuspended in 1 N NaCl with stirring and again allowed to settle followed by decanting.

This procedure was repeated 2-3 times before finally filtering by suction through Whatman #1 filter paper (W.R. Balston Ltd., England) on a Buchner funnel. The cellulose cake was resuspended in 1 N HCl, filtered immediately by suction and washed with distilled water until the pH was neutral. The ion exchanger was then washed and equilibrated to the desired pH and molarity with the starting buffer. The thick cellulose buffer mixture was homogenized in a Waring blender and trapped air bubbles were evaculated with a vacuum pump. The homogeneous cellulose slurry was then poured into a glass column, which was partially closed by a glass wool plug above glass beads, and allowed to settle to 4-5 cm height at the bottom of the column with the outlet closed. The outlet was then opened and excess buffer allowed to escape. More slurry was added through a 5-8 cm column of buffer to avoid any disturbance of the packed bed. External pressure was applied by pressing down on a rubber stopper closing the top of the column to pack the last 15 cm or so of the column. When the column was packed, a piece of filter paper with slightly smaller diameter than the glass column was laid over the exposed cellulose to protect the cellulose surface and to provide a flat surface for sample application. The DEAE cellulose column was packed at R.T. under atmospheric pressure and ran under a pressure greater than atmospheric pressure at a rate of 30 to 40 mls per hour.

IX. Sephadex G100 and G200 Gel Filtration

Both Sephadex G100 and G200 columns were packed according to the instructions provided by Pharmacia Ltd. (Montreal, Canada). The gels were swollen in 0.15 M borate saline pH 8.0 at R.T. for three days or five hours at 60°C. The gels were added gradually to an excess of buffer with mixing to facilitate dispersion of the beads. Decanting and stirring were carried out intermittently during the swelling procedure. Trapped air was removed by a vacuum pump before the gel-buffer (1:1 in volume) mixture was used for packing. Approximately 15-20 cm of buffer was poured into a vertically mounted Sephadex laboratory column and air bubbles trapped in the polyethylene disc were removed by forcing the buffer back and forth through the disc by a syringe attached to the outlet tubing. The outlet was then closed and the gel-buffer slurry slowly poured down the wall of the column until it reached the top. The outlet of the column was always kept at the same level with the top of the gel slurry. When approximately 10 cm of the bed had settled, the outlet was slowly lowered to maintain a pressure equal to 1 cm. As the packed bed rose, the outlet tubing was lowered to maintain an optimum pressure equal to one-tenth of the packed bed height. To avoid formation of boundaries, the excess buffer was removed and another portion of the gel slurry was added before the previous portion had settled completely. After the column was packed, the upper surface

of the bed was protected by a sample applicator. The column was allowed to equilibrate for 24 hours at R.T. at 10-15 cm pressure at a rate of about 20 ml/hour. 5 mg of blue dextran in 3 ml of buffer was passed through the column to determine the void volume and to ensure the homogeneity of the packing. In actual experimental situations, the samples were allowed to filter through at a rate of 5-10 ml per hour under a pressure of about 2-3 cm.

The G100 column was packed under atmospheric pressure. Care was again taken not to create boundaries by pouring the well mixed gel slurry slowly down the column before previous portions had settled completely. The column was generally ran at greater than atmospheric pressure at a flow rate of 30-40 ml per hour.

X. Immunoelectrophoresis

The immunoelectrophoretic method of Grabar and Burtin (420) was adopted. 2 ml of 0.5% melted agar (Difco, Noble) in distilled water was used to coat each 25 x 76 cm glass slide which was then air dried at 80°C for 4 hours. These slides were subsequently layered with 3 ml of 2% melted agar in 0.025 M barbital buffer pH 8.5 and allowed to solidify. The required wells and troughs were cut and the test materials placed in an electrophoretic apparatus and subjected to a 14 ma current approximately 70-75 volts for $3\frac{1}{2}$ hours. After removal from the apparatus and filling the troughs with the appropriate detecting antisera, the slides

were placed in a moist chamber at R.T. overnight to allow the development of the precipitin lines. They were then washed in 0.15 M saline for 24 hours, desalted in distilled water for 8 hours and then dried overnight by placing filter paper on the slides. The dried slides were stained with amido black (1 gm/1000 ml sodium acetate buffer) for 10 minutes, decolorized in an acetic acid: methanol: water solution (20:980:300 by volume) for another 10 minutes and then air dried.

XI. Ouchterlony Gel Diffussion

Slides coated as for immunoelectrophoresis were layered with 3 ml of 1.5% melted agar in 0.15 M saline. After allowing the gel to solidify in a moist chamber, the wells were cut with a hole puncher and then filled with antigen or antibody solution in such a manner that the desired pattern of precipitin lines would be obtained. The slides were then placed in a moist chamber at R.T. overnight for the precipitin lines to develop. Staining and washing were performed as described for immunoelectrophoresis the next morning.

XII. Immunoadsorbents

1. Bis-diazotized Benzidine (BDB) Method

The BDB reagent was prepared according to the method given in the Handbook of Experimental Immunology

(421).

Benzidine, (Hartman-Leddon Co., Philadelphia, Pa.) in 230 mg aliquots, was dissolved in 45 ml of 0.2 N HCl and cooled in an ice bath. 175 mg of $NaNO_2$ (J.T. Baker Chemical Co., Phillisburg, N.J.) dissolved in 5 ml of distilled water was cooled and added to each aliquot of benzidine over a period of 1 minute. The reaction was allowed to proceed for 30 minutes with stirring at 5 minute intervals. The resulting stock solution was stored as 2 ml aliquots in sealed ampules at -20°C.

The method described by Bernier and Cebra (422) was used to aggregate all proteins. To 5 ml of 1/15 dilution of the stock solution prepared above was added 15 mg of the appropriate antigen dissolved in 5 ml of 0.1 M phosphate buffer pH 6.8 and the solution allowed to stand at R.T. for 5 hours. The resultant aggregate was washed 3x with buffer, 2x with 0.1 M glycine-HCl buffer pH 2.5 and finally neutralized with 0.2 M phosphate buffer pH 7.2. The aggregates were recovered after each washing by centrifuging at 3000 rpm for 5 minutes at R.T.. 1 ml of normal rabbit serum (NRS) was added to the aggregate and the suspension gently stirred at R.T. for 1-2 hours followed by continuous stirring at 4°C overnight. The aggregate was then recovered by centrifugation and washed 2x with 0.2 M phosphate buffer, once with glycine-HCl buffer, neutralized with 0.2 M phosphate buffer and stored at 4°C until use. The NRS was added to fill up any potential sites on the

aggregate capable of nonspecifically adsorbing proteins.

2. Ethyl Chloroformate (Tear Gas) Method

Proteins were also aggregated by ethyl chloroformate according to the method of Avrameas and Ternynck (423). To 100 mg of protein antigen in 3 ml of 0.2 M acetate buffer pH 4.5 was added 0.2-0.3 ml of ethyl chloroformate (K & K Laboratories Inc., Plainview, N.Y.) over a period of 1 minute with efficient stirring. The mixture was allowed to stir for an additional 15 minutes and then allowed to stand for an hour while the pH was kept between 4.5-5.0 throughout this period. The aggregate which resulted was washed 3x with acetate buffer, 2x with 0.1 M glycine-HCl buffer pH 2.5 and neutralized with 0.2 M phosphate buffer pH 7.2. The aggregate) was further treated with NRS and washed as described for the BDB aggregates before use.

3. Cyanogen-bromide (CNBr) Activated Sepharose 4B Method

CNBr-activated Sepharose 4B was conjugated according to the instructions provided by Pharmacia Ltd., Canada. 1 gm of the activated sepharose was washed 4x with 40 ml of 10^{-3} M HCl followed by 40 ml of 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl pH 8.0. 15-30 mg of the protein to be coupled was dissolved in 5 ml of the $NaHCO_3$ buffer, mixed with the gel and the mixture was rotated end over end or shaken continuously but gently for 2 hours at R.T. or overnight at $4^{o}C$. Unbound material was washed away with

coupling buffer and any remaining active groups were reacted with 1 M ethanolamine pH 8.0. Three washing cycles were used to remove non-covalently adsorbed protein, each cycle consisting of a wash at pH 4 with 0.1 M actate buffer containing 1 M NaCl followed by a wash at pH 8.0 with 0.1 M borate buffer containing 1 M NaCl. The conjugated sepharose was packed over glass beads and glass wool in a 10 ml plastic syninge and stored at $4^{o}C$ in 0.15 M borate saline buffer, pH 8.0 containing a drop of toluene.

XIII. Purification of Antibodies by Immunoadsorbents

1. By Antigen Aggregates

10 ml of rabbit antiserum was mixed with 50-100 mg of the aggregated antigen and the suspension was gently stirred initially for 2 hours at R.T. and then overnight at $4^{o}C$. The aggregate was then separated from the serum by centrifugation at 10,000 rpm for 15 minutes and washed three times with 0.15 M phosphate buffered saline pH 6.4 at $4^{o}C$. The antibodies were eluted by suspending the aggregate in 0.1 M glycine-HCl buffer, pH 2.5 for 1 hour at $4^{o}C$, followed by centrifugation at 10,000 rpm for 15 minutes at $4^{o}C$. The supernatant containing the antibodies was recovered and neutralized immediately. The aggregate was also neutralized and the process of elution was repeated to recover as much antibody as possible from the antiserum. The specificity of the isolated antibodies was examined by the Ouchterlony

technique and the concentration of specifically precipitable protein was found to be between 80 and 90%. A stepwise elution procedure from DEAE-cellulose column was employed to further purify the antibodies obtained.

2. By Conjugated Sepharose

CNBr activated Sepharose, conjugated with the appropriate antigen, as described in section XII-3, was washed with 25 ml of fresh 0.15 M borate saline buffer pH 8.0 before each use. 5 ml of rabbit antiserum was then filtered through the sepharose column in a period of 1 hour at R.T. and the effluent collected. The Sepharose was rinsed rapidly with 100 ml of borate buffer and eluted with 5 ml of 0.1 M glycine HCl buffer pH 2.5, within a span of 15 minutes, followed by another 5 ml of borate buffer. The eluant was immediately neutralized with 1 M NaOH while the sepharose was neutralized with more borate buffer. Both the original effluent and the subsequent eluate were examined by the Ouchterlony technique for specific antibody activity. The concentration of specifically precipitable protein in the eluate was found to be at least 80%.

XIV. Pepsin Digestion of Antibodies

The $F(ab')_2$ fragments of purified rabbit antimouse Ig and anti-BSA antibodies were obtained following the pepsin (Worthington Biochemical Co., Freehold, N.J.) digestion method of Nisonoff et al (424) and Utsumi and Karush (425).

Pepsin digestion was carried out using a pepsin to protein ratio of 2:100. The pepsin was dissolved in an appropriate volume of 0.1 M acetate buffer pH 4.0 and the antibody solution which has been previously equilibrated in the same buffer. After about 20 minutes at 37°C, the pepsin solution was rapidly added to the antibody solution and the mixture thoroughly mixed and allowed to incubate for 5 hours at 37°C. The digestion was terminated by neutralization to pH 8.0 with 1 N NaOH and the sample applied on a Sephadex G100 column (23 x 970) equilibrated with 0.15 M borate buffered saline pH 8.0. $F(ab')_2$ fragments were recovered from the 5S peak and found to form a precipitin line with the homologous antigen by the Ouchterlony technique.

XV. Coating of Sheep Red Blood Cells

SRBC to be coated with protein were formalinized according to the method of Wede (426). Collected in Alsever's solution, the sheep erythrocytes were washed 3x with 0.15 M saline pH 7.5. Equal volumes of a 8% SRBC in saline solution and a 3% formaldehyde solution adjusted to pH 7.5 with 0.1 N NaOH were incubated at 37°C for 24 hours, after which, the cells were washed 4x in distilled water at neutral pH and then stored as a 10% sodium azide or formalin at 4°C for up to 6 months. From this, a 2% suspension was prepared for tanning and coating according to the method described by Herbert (421). After washing 3x with 0.15 M phosphate buffered saline pH 6.4, the

formalinized red cells were incubated with 2 ml of a 0.0025% tannic acid (Baker Chemical Co., Phillisburg, N.J.) solution for half an hour at 37°C. The cells were washed once and left at 4°C overnight. The cells were then resuspended in 2 ml of buffer containing 0.14 mg/ml of BSA and the suspension incubated at 37°C for an hour. The cells were washed 3x with buffer and then finally suspended in 1.5 ml buffer containing a drop of a 2.5% solution of human serum albumin. The resulting cell suspension was approximately 2.5% by volume.

XVI. Hybrid Antibody Preparation

Hybrid antibody was prepared as described by Paraskevas et al (97 , 98). The purified anti-Ig and anti-BSA antibodies were individually pepsin digested as described previously and $F(ab')_2$ fragments purified by filtration on a G100 Sephadex column. The method of Nisonoff and Rovers (427) was followed in the reduction and reoxidation of the $F(ab')_2$ fragments. Equal amounts of anti-BSA and anti-Ig $F(ab')_2$ fragments were mixed and reduced with 0.015 M 2-mercaptoethylamine hydrochloride (Matheson Colman & Bell, Norwood, Ohio) under N_2 for 1 hour at 37°C to obtain univalent F(ab') fragments. The reducing agent was removed immediately by passage of the sample through an AG-50W-X4 cation exchange resin (Bio-Rad Laboratories, Mississauga, Ontario). The eluate was reoxidized by bubbling molecular O_2 through the univalent antibody fragment solution for 2

hours at R.T. The divalent $F(ab')_2$ fragments were recovered by passing the sample through G100 Sephadex column.

The $F(ab')_2$ fragments obtained contains 3 recombinants, namely bivalent anti-Ig, bivalent anti-BSA and the hybrid anti-Ig - anti-BSA. A two step absorption elution procedure was employed in order to isolate the hybrid recombinant. First the reoxidized material was absorbed with a BSA aggregate or BSA conjugated Sepharose, and eluted with 0.1 M glycine-HCl buffer pH 2.5. The acid eluant, containing the bivalent anti-BSA and the hybrid recombinants was immediately neutralized and absorbed with a mouse Ig aggregate. This aggregate selectively retains the hybrid recombinant which was subsequently eluted with the acid buffer and neutralized. Ouchterlony gel diffusion confirmed the presence of only the hybrid molecules in the eluate from the second aggregate. The normal recovery of hybrid antibody by these procedures was around 20%. Hybrid antibodies used in this study were antimouse Ig - anti bovine serum albumin (αMIg - αBSA). The antimouse Ig used reacted with heavy chains of mouse IgG and IgF as well as with mouse light chains.

XVII. Reverse Immunocytoadherence (RICA) Technique

This assay of detecting surface Ig was performed in accordance to the method described by Paraskevas et al (97,98). The technique utilizes a 5S hybrid antibody with one binding site specific for mouse Ig and the other for BSA.

An indicator system consisting of SRBC coated with BSA enables the formation of rosettes of SRBC around a centrally located lymphocyte containing surface Ig. This was made possible by the hybrid antibody which attaches, through its anti-BSA and anti-MIg sites, the indicator cells to the Ig positive lymphocyte respectively.

1. Preparation of Single Cell Suspensions

Inbred Balb/c mice 6-10 weeks of age were sacrificed with chloroform and bled through cardiac puncture. Their spleens and/or thymuses were rapidly removed and transferred to cold Hank's balanced salt solution (HBSS, Microbiological Associates Inc., Bethesda, Maryland) in an ice bath. A single cell suspension was obtained by needle or forcep teasing of the spleen followed by passage through a stainless steel mesh (200/inch). The cells were washed 2x with HBSS and recovered by centrifugation at 700 rpm for 7 minutes. After appropriate dilution with HBSS, a total cell count was made in 2% acetic acid with a hemocytometer while the trypan blue exclusion technique was employed to enumerate the number of viable cells.

2. RICA Assay

30 µl of a BSA coated SRBC solution were added to 0.7-1.0 x 10^6 spleen cells usually contained in 0.1 ml HBSS (approximately 100 SRBC per spleen cell). 50 µg of hybrid antibody in 30 µl of buffer was added to this mixture and

gently but thoroughly mixed. The mixture was incubated undisturbed overnight at $4^{o}C$. In all experiments, controls consisting of normal spleen cells with hybrid antibody and test cells without hybrid were always prepared under similar conditions.

The test was read by counting cells and rosettes using Bellco slides (Bellco Glass Inc., Vineland, N.J.) with 20 mm square chambers. The settled cells were gently brought into a homogeneous suspension and then carefully introduced into the chamber using Pasteur pipettes. The chamber was covered by a 22 mm square glasscoverslip and the cells were allowed time to settle. A phase contrast microscope (Carl Zeiss) with a 40x objective was used to count the total number of rosette forming cells (RFC). Only lymphoid cells surrounded by a minimum of four SRBC were scored as a rosette. Usually 1000 spleen cells were counted and the test result expressed as the number of RFC per 1000 spleen cells.

XVIII. Enumeration of Theta Antigen Positive T Lymphocytes by Cytotoxicity Test

1. Preparation of Anti-theta Antiserum ($\alpha\theta$)

This antiserum was prepared in AKR mice against C3H thymocytes as described by Reif and Allen (428). AKR mice were given weekly i.p. injections of 1×10^{7} washed C3H thymocytes for 6 weeks. 10 days after the last injection the mice were killed and bled by cardiac puncture. The

serum obtained was absorbed at 4°C for 30 minutes with packed red blood cells from C3H and Balb/c mice and decomplemented by heat inactivation at 56°C for 30 minutes. The absorbed and decomplemented serum was stored at -90°C in small aliquots until use. Only preparations which killed over 90% of Balb/c and C3H thymocytes at a dilution of 1:64 and proved non-cytotoxic to bone marrow cells were retained for use.

2. Anti-theta Cytotoxicity Test

The method followed was that of Takahashi et al (429). 2-3 x 10^6 spleen cells were incubated with the antitheta antiserum (1:6 dilution) and guinea pig complement (1:3 dilution; Pentex) for 45 minutes at 37°C. Viable cells were determined by the trypan blue exclusion method. Spleen cells incubated only with antiserum served as control. The percent cytotoxicity was determined using the following formula:

$$\% \text{ cytotoxicity} = \frac{Vc - Vt}{Vc} \times 100$$

V_C = Viable count of control (Antiserum)

V_T = Viable count of test (Antiserum + complement)

XIX. Induction of Immunologic Unresponsiveness to HRBC or SRBC

Tolerance to HRBC or SRBC was induced in Balb/c mice according to the method described by Anderson et al (417).

The tolerogen horse erythrocyte hemolysate supernatant (HHS) was prepared as described in section III. Animals were injected with 0.4 ml of HHS (roughly equivalent to supernatant from 2 x 10^8 HRBC) i.p. daily for 5 days and allowed to rest for 2 days before the challenging dose (5 x 10^8) of HRBC was given i.p.. The IgM and IgG antibody response to HRBC was determined 4 and 6 days after challenge respectively using the Plaque forming cell (PFC) assay of Cunningham and Szenberg (430).

XX. Plaque Forming Cell (PFC) Assay

PFC assay was performed according to a slightly modified method of that of Cunningham and Szenberg (430). 'Cunningham' slides were prepared by laying 3 pieces of double-sided tape (Scotch brand No. 410) each $\frac{1}{4}$ inch wide across a clean microscope slide (75 x 25 mm) dividing it into two equal areas. Another microscope slide of same dimensions was pressed firmly on to the taped slide so as to create two thin chambers, with a depth equivalent to the thickness of the tape, between the two slides.

A mixture is made, of the lymphoid cells under test (30 µl of a 10-15 x 10^6 viable cells per ml solution), together with 30 µl of freshly thawed guinea pig complement, and 30 µl of erythrocytes of the type used for immunization (at a concentration of 2.5 x 10^9 red cells/ml). For indirect PFC, 30 µl of rabbit IgG anti-mouse Ig of a dilution previously found to give maximal number of PFC

were added to the mixture. After mixing, the mixture was introduced into the chambers of Cunningham slides with a Pasteur pipette. An appropriately diluted red cell solution was used to fill up any unoccupied space in the chambers. The chambers were sealed with paraffin wax and the slides incubated horizontally at 37°C for 45 - 60 minutes. PFC were counted under a light microscope (Zeiss) using low power objective. Results were usually expressed as PFC per 10^6 cells or PFC per spleen. In general, reproducibility of the assay is quite acceptable.

XXI. Phytohemagglutinin (PHA) Stimulated Lymphocyte Transformation: Tritiated Thymidine Uptake

In vitro activation of lymphocytes by the plant mitogen, PHA, was measured by the uptake of tritiated thymidine as described by Adler et al (431) and Stobo et al (396) but with some modifications. Animals were sacrificed and single cell suspension prepared aseptically from the spleen in Roswell Park Memorial Institute Medium 1640 (RPMI 1640). The medium was supplemented with 100 µg of streptomycin and 100 units of penicillin per ml of medium and 10% Fetal Calf Serum (FCS) (all from Grand Island Biological Co., Grand Island, N.Y.). Routinely, 12 x 75 mm culture tubes (Falcon Plastics Oxnard, Calif.) were used and 1.5 to 2.0 x 10^6 viable spleen cells were cultured in quadruplicate in 0.9 ml of medium. 0.1 ml of 1/20 diluted, purified PHA (Wellcome Reagents Ltd., Beckenham, England) or 0.1 ml of medium was added and the cultures incubated in a humidified

atmosphere of 5% CO_2, 95% air at 37°C for 48 to 72 hours. 1 μCi of tritiated thymidine (H^3 - Tdr; 2 Ci/mM specific activity, New England Nuclear, Boston, Mass.) was added for the last 20 hours of culture.

At harvest, the tubes were spun down at 3000 rpm at 4°C and washed 3x with cold saline. The cells were resuspended in 0.1 ml of cold saline and transferred to scintillation vials. The culture tubes were then rinsed with another 0.1 ml of cold saline which was also transferred to the corresponding vials. To each vial was added 1 ml of NCS tissue solubilizer (Amersham/Searle, Arlington Heights, Ill.) and the solution thoroughly mixed and allowed to incubate for 30 minutes at 37°C. 10 ml of cold scintillation fluid (0.105 gm 1,4-bis-2-(5-phenyloxaxolyl)-benzene (POPOP), 4.21 gm 2,5-diphenyloxazole (PPO) in 1 litre of T330 toluene, all from Fisher Scientific Corp., Fairlawn, N.J.) was dispensed into each vial and the radioactivity determined by a Tricarb Liquid Scintillation Spectrometer (Packard Instruments, Downers Grove, Ill.). The results were expressed as counts per minute (cpm) and each value recorded in the text represents the mean of quadruplicate cultures. The stimulation index was defined as the ratio of the mean cpm in the presence and absence of PHA.

XXII. Cell Mediated Cytotoxicity (CMC) Assay

The assay method was essentially that of Thorn et al

(432) with some modifications.

1. Labelling of Target Cells with ^{51}Cr

Target cells (EL-4 lymphoma cells recovered from the peritoneal cavity 6-8 days after transplantation in 6-8 week old C57 B1/6J mice) were washed in RPMI 1640 medium (+ Penicillin and Streptomycin + 10% FCS) and made up to a concentration of 10×10^6 viable cells/ml. 0.8 ml of this solution containing 8×10^6 tumor cells were mixed with 0.2 ml ^{51}Cr (200 μCi, Amersham/Searle, Arlington Heights, Ill.) and incubated at 37°C for 45 minutes with constant shaking. The cell were washed 5x in medium and adjusted to 4×10^5 cells/ml.

2. Preparation of Effector Spleen Cells

Single cell suspensions were prepared in 3 ml of cold RPMI 1640 (+ 10% FCS + Penicillin and Streptomycin) medium and allowed to stand a few minutes for dead cells to settle out. The supernatant was removed, spun at 200 g for 10 minutes at R.T. and washed 2x with cold medium. Solutions containing appropriate concentrations of effector cells were prepared in medium.

3. CMC Assay

Cultures were always set up in triplicate in U type microtiter plates (Cooke Engineering, Alexandria, Va.). 0.05 ml medium containing 2×10^4 target cells were dispensed into each well followed by 0.20 ml of effector

cell solutions containing cell numbers such that effector/target ratios between 10 to 100 were obtained. After centrifugation at 1300 rpm for 2 minutes, the microtitre plates were covered with plastic film (Cooke Engineering, Alexandria, Va.) and incubated at 37°C for 16 hours. At the end of the incubation period, the microtitre plates were spun at 200 g for 10 minutes and 100 µl of cell free supernate removed to determine the amount of radioactivity present by means of a gamma counter (Packard 5230 Auto-gamma Scintillation Spectrometer). The percentage corrected lysis was calculated with the expression (E - C)/(T - C) where C = mean counts per minutes (cpm) in the supernate of control wells containing normal spleen cells; E = cpm in the supernate of experimental wells and T = total releasable radioactivity, i.e. mean cpm in the supernate of wells in which 100% target cell lysis had been induced by freezing and thawing 4 times.

XXIII. Delayed Type Hypersensitivity (DTH)

1. Sensitization

Adult Balb/c mice were immunized with the antigen emulsified in FCA intracutaneously or subcutaneously in the right hind footpads in a volume of 0.02 ml. The dose of antigen used was that previously determined to elicit a maximal response.

2. Footpad Assay of DTH

The mercury immersion method described by Axelrad (433) for rats was adopted. Essentially the method depended on the principle that an object immersed in a fluid displaces its own volume. If the object (of volume V ml) has a lower specific gravity (So g/ml) than that of the fluid (Sf g/ml), the pressure (P_g) needed to achieve total immersion is given by the formula $P = (S_f - S_o)\ V$. Since $(S_f - S_o)$ is a constant, any change in V will be reflected in a change in P. The method was noted to detect as small a volume change as 0.02 ml. A top-loading, single pan balance with an optical scale calibrated in 10 mg divisions was used (Sartorius 2250). The reading of a small mercury filled beaker was first obtained and then the additional deflection produced by immersing the mouse foot, to the level of a line drawn in the groove immediately distal to the lateral malleolus, was recorded. Readings, usually of the left foot, were made before antigen challenge and 20 - 24 hours after challenge determine the degree of swelling. Each reading represents the mean of 4 - 5 measurements. Results were expressed as percentage increase in footpad volume (or % swelling) obtained by the formula :

$$\% \text{ swelling} = \frac{W_A - W_B}{W_B} \times 100$$

W_A = Wt. after challenge

W_B = Wt. before challenge

XXIV. Passive Hemagglutination Test

A slightly modified version of the method described by Herbert (421) was used. The test was carried out in capillary tubes which hold a series of doubling dilutions of the antiserum to be examined in 0.01 ml volume of 0.15 M phosphate buffered saline pH 6.4. To each capillary tube, 0.01 ml of a 2.5% SRBC suspension coated with antigen was added. The capillary tubes were inverted and allowed to stand at R.T. for 30 minutes. The agglutination reaction was observed with a magnifying glass; a positive test being indicated by the aggregation of SRBC while a smooth column of SRBC persists in case of a negative reaction.

XXV. Irradiation and Adoptive Transfer of Spleen Cells

Adult Balb/c mice were sublethally irradiated (550R) using an Eldorado A^{60} Cobalt therapy machine (Atomic Energy of Canada Ltd.). Irradiated animals were usually reconstituted immediately, with 20 x 10^6 viable spleen cells intravenously in 0.4 ml of sterile Eagles' Minimum Essential Medium (GIBCO) through tail veins followed 45 - 60 minutes later with i.p. injection of antigen if necessary.

XXVI. Spleen Cell Suspensions

1. Normal Adult Balb/c Mice

The RICA (Materials and Methods Section XVII-2) and/or

anti-theta (Materials and Methods Section XVIII-2) were used to detect the proportion of Ig and/or θ carrying lymphocytes respectively in mouse spleen obtained under the conditions specified:

i. 6 and 18 hours, 7, 15 and 29 days after injection with 5×10^8 HRBC.

ii. 6 hours after injection with 5×10^8 HRBC, 5×10^8 CRBC, 5×10^8 SRBC, 15×10^6 frozen and thawed C_3H spleen cells, 250 μg fe/FCA, 250 μg EA/FCA and 250 μg FIB/FCA.

iii. 6 and 30 hours after pre-treatment with 1 x 0.4 ml of tolerogen and 30 hours after pre-treatment with 3 x 0.4 ml of tolerogen.

iv. 6 hours after challenge with 5×10^8 HRBC given 24 hours after pre-treatment with 1 x 0.4 ml or 3 x 0.4 ml tolerogen.

The plaque forming cell assay (Materials and Methods Section XX) was performed on spleens obtained as follows:

i. 2, 3, 4, 5, 6, 7 and 14 days after 5×10^8 HRBC.

ii. 4 and 6 days following a challenging dose of 5×10^8 HRBC given 2 days after pre-treatment with 1x, 2x, 3x, 4x or 5x 0.4 ml of tolerogen.

iii. 4 and 6 days following immunogenic doses of 5×10^8 HRBC and 5×10^8 CRBC and 4 and 7 days after 5×10^8 SRBC were administered.

iv. 4 and 7 days after challenge with 5×10^8 SRBC of mice pretreated with UM10 filtrate of 6 days

hyperimmune serum.

v. 4 and 6 days after challenge with 5 x 10^8 HRBC of mice pretreated with UM10 filtrate of serum or whole serum of tolerant mice or with saline or with UM10 filtrate of normal mouse serum.

vi. 4 and 6 days after challenge with 5 x 10^8 HRBC of mice sublethally irradiated and reconstituted with 20 x 10^6 viable normal spleen cells.

vii. 4 and 6 days after challenge with 5 x 10^8 HRBC of sublethally irradiated 20 x 10^6 normal spleen cell reconstituted mice pretreated with 6 day UM10 filtrate from normal or tolerant mice with or without challenge with HRBC (5 x 10^8).

viii. 7 days after immunogenic stimulation with 5 x 10^8 SRBC of sublethally irradiated mice reconstituted with 20 x 10^6 viable cells from 6 hour immune spleens or from normal spleens.

2. HRBC Tolerant Adult Balb/c Mice

Ig and θ carrying lymphocytes were detected by RICA and anti-theta cytotoxicity tests (Materials and Methods Section XVII-2) (Materials and Methods Section XVIII-2) in spleens obtained as specified below:

i. 2, 6 and 16 days after last administration of tolerogen.

ii. 6 hours after administration of 5 x 10^8 HRBC given 2, 7, 14, 22, 32 and 70 days after last injection of

tolerogen.

iii. 7 days following administration of 5×10^8 HRBC, 5×10^8 CRBC and FIB/FCA (250 µg) given 2 days after last tolerogen injection.

iv. 6 hours, 1, 4, 6, 7 and 15 days after immunogenic challenge with 5×10^8 HRBC given 2 days after last injection of tolerogen.

v. 6 hours after challenge with 5×10^8 HRBC, 5×10^8 CRBC, 5×10^8 SRBC, 15×10^6 frozen and thawed C_3H spleen cells. 0.1 mg BCG, 250 µg Fe/FCA, 250 µg FIB/FCA, 250 µg EA/FIB, 0.2 ml saline and 0.2 ml saline-FCA mixture (1:1) all given 2 days after last injection of tolerogen.

PFC assays (Materials and Methods Section XX) were done on spleen cells of animals specifically treated as follows:

i. 5 days after 5×10^8 HRBC were given 2, 21, 32 or 70 days after last injection of tolerogen.

ii. 4 and 7 days after challenge with 5×10^8 SRBC or 4 and 6 days after 5×10^8 HRBC or 5×10^8 CRBC given 2 days after last tolerogen injection.

iii. 4 and 6 days after challenge with 5×10^8 HRBC of sublethally irradiated mice reconstituted with 20×10^6 viable tolerant spleen cells.

iv. 4 and 7 days after challenge with 5×10^8 SRBC of SRBC tolerant mice 2 days after last tolerogen were given.

v. 7 days after immunogenic challenge (5×10^8 SRBC or 5×10^8 HRBC) of sublethally irradiated mice reconstituted with 20×10^6 viable HRBC tolerant spleen cells.

vi. 7 days after immunogenic challenge with 5×10^8 SRBC of sublethally irradiated mice reconstituted with 20×10^6 viable spleen cells obtained from tolerant mice stimulated with 5×10^8 SRBC 6 hours previously.

XXVII. Treatment of Normal Spleen Cells in Vitro

Spleen cell suspensions were prepared and washed in cold Hank's balanced salt solution. After counting, 5×10^6 normal spleen cells were dispensed into culture tubes (Falcon Plastics, Oxnard, Calif.) in 0.2 ml of HBSS and incubated at 37°C for 30-45 minutes with 0.2 ml of the following:

1. Serum collected from normal mice 6 hours after challenge with 5×10^8 HRBC, 5×10^8 CRBC, 250 µg of either FIB or EA or Fe in FCA, saline.
2. Serum collected from HRBC tolerant mice 6 hours after challenge with 5×10^8 HRBC, 5×10^8 CRBC, 5×10^8 SRBC, 15×10^6 C_3H spleen cells, 0.1 mg BCG, FCA, saline, and 250 µg of either FIB or EA or Fe in FCA.
3. Serum collected from HRBC tolerant mice 2 days after the final injection of HHS.

After incubation, the cells were washed 3x with cold Hank's balanced salt solution and finally resuspended in

0.5 ml of Hanks. 0.1 ml aliquots of this suspension (approximately 0.8 - 1.0 x 10^6 cells) were used to set up RICA and anti θ cytotoxicity assays.

XXVIII. Treatment of Tolerant Spleen Cells in Vitro

Tolerant mice were sacrificed and bled 2 days after last injection of tolerogen and single cell suspensions prepared routinely (Materials and Methods Section XVII-1). 5 x 10^6 tolerant spleen cells, contained in 0.2 ml HBSS, were incubated at 37°C for 30 - 45 minutes with serum collected from normal mice 6 hours after challenge with 5 x 10^8 HRBC or 5 x 10^8 CRBC or 250 µg FIB/FCA or 250 µg EA/FCA, and then washed 3x with cold Hank's balanced salt solution before making up to 0.5 ml with Hank's. 0.1 ml aliquots of this cell suspension (approximately 0.8 -1.0 x 10^6 cells) were used to set up RICA and anti θ cytotoxicity assays.

XXIX. In Vitro Preparation of IACF

Thymocytes, 1 x 10^7 or 4 x 10^7/ml, were suspended in Hank's solution and were exposed for 30 minutes at 37°C to 100 µg of mycobacteria (BCG Connaught Laboratories, Toronto). After washing (x3), they were cultured in fresh medium for 3 more hours and the supernates were collected by removing the cells with centrifugation. As controls, we used supernates from cells treated in a similar way in abscence of BCG. The ability of supernates to induce cytophilic Ig was tested by

incubation of 0.5 ml of supernate with 1 x 10^7 normal spleen cells for 30 minutes at 37°C in the presence of normal mouse serum and antigen. After incubation, the cells were washed with Hank's solution and used in RICA tests at a concentration of 2-3 x 10^6/ml.

In vivo preparation of IACF consists of injecting 0.2 ml of FCA/saline emulsification in 1:1 ratio intraperitoneally into mice and collecting blood from these animals 6 hours later. Sera obtained were tested for their ability to induce cytophilic Ig by incubating 0.2 ml of the serum with 5 x 10^6 normal spleen cells at 37°C for 30 minutes in the presence of antigen. Cells were washed (3x) after incubation and used in RICA tests as above.

Only supernates and 6 hour FCA serum with tested cytophilic activity were used for experiments described in Results, section XXIV.

XXX. Preparation of Serum UM10 Ultrafiltrates

One millilitre of serum is diluted with equal volume of Hank's solution and filtered through a Diaflo UM10 membrane (Amicon, Lexington, Massachusetts). One millilitre of filtrate was collected and will be referred to as UM10 filtrate.

XXXI. Statistical Analysis

All statistical analyses were performed using a two sample student t test and an Olivetti programma 101 electronic desk computer.

RESULTS

RESULTS

I. Ig BEARING LYMPHOCYTES IN SPLEENS OF NORMAL ADULT BALB/C MICE

The RICA technique was used to determine the number of Ig bearing cells in spleens of normal adult Balb/c mice. Spleen cells were obtained by the method described in Materials and Methods Section XVII-1 and the RICA assay set up according to the description in Materials and Methods Section XVII-2. An antimouse Ig-antiBSA hybrid antibody specific for all classes of mouse Ig and an indicator system consisting of BSA coated SRBC was used throughout the RICA assays represented in this thesis. Of necessity, different batches of hybrid antibody and indicator red cells were used during the course of the work. However, the reagents used in each particular experiment were from the same batch. In addition, each experiment always included a control consisting of normal spleen cells subjected to identical reagents under similar experimental conditions. Another routine control in RICA assays involved spleen cells with indicator red cells but no hybrid antibody. This was done to monitor non-specific rosettes resulting from non-specific adherence of the indicator cells to the lymphocytes. Experimental results were discarded when such 'background' values exceeded 15 rosettes per 10^3 spleen cells. Most often, the non-specific rosettes numbered around 8-10 per 10^3 spleen cells counted. The mean number of Ig

bearing lymphocytes or RFC (± standard error) in 57 randomly sampled normal spleens was found to be 305±3 per 10^3 spleen cells, i.e. roughly 30% spleen cells exhibit surface Ig.

II. θ BEARING LYMPHOCYTES IN SPLEENS OF NORMAL ADULT BALB/C MICE

The percent of θ positive cells in the spleens of normal Balb/c mice were enumerated by the cytotoxicity assay outlined in Materials and Methods section XVIII-2. Tests and controls were always subjected to identical reagents and experimental conditions in each individual experiement. Random sampling of 31 normal spleens yielded a mean percentage of 29±1 for their θ positive population.

III. TOTAL NUMBER OF SPLEEN CELLS IN NORMAL ADULT BALB/C MICE

Single cell suspensions of normal spleens were routinely prepared and the total number of nucleated cells determined using a hemocytometer. The mean value found was $1.36 \pm 0.32 \times 10^8$.

IV. Ig CARRYING LYMPHOCYTES IN SPLEENS OF BALB/C MICE 6 HOURS AFTER IMMUNIZATION WITH ANTIGEN

The number of Ig carrying cells was determined in spleens obtained from adult Balb/c mice 6 hours after administration of a variety of antigens. A marked increase in the cells exhibiting surface Ig was found. Such an increase amounted to 8 to 10% of

total spleen cells or approximately 25% of Ig carrying lymphocytes before immunization and was highly statistically significant ($p < .001$). The observed increase was not limited to any particular antigen and could be elicited by heterologous erythrocytes, allogeneic cells and soluble protein antigens emulsified in FCA (Table I). That soluble proteins without FCA do not elicit a 6 hour increase in Ig + spleen cells has been documented previously in our laboratory (1). The same workers have also provided conclusive evidence that the increase in Ig + cells was:

1/ not due to proliferation of existing RFC in view of the similarity in total spleen count of immunized and non-immunized animals (see also Section V of Results) and the lack of time for generation of new cells as well as the large numbers of cells involved (1).

2/ not due to immigration of RFC cells since the same increase is reproducible in vitro by incubating normal spleen cells with serum collected 6 hours after antigenic stimulation (1) (also Table II).

3/ due to the uptake of cytophilic γG globulin likely complexed to antigen, by T cells (2).

V . θ CARRYING LYMPHOCYTES IN SPLEENS OF BALB/C MICE 6 HOURS AFTER ADMINISTRATION OF ANTIGEN

Spleens were removed from mice 6 hours following challenge with various antigens and examined for their content of

TABLE I

Ig CARRYING SPLEEN CELLS SIX HOURS AFTER INJECTION OF ANTIGEN [a]

Antigen	Dose	Ig+ cells per 10^3 spleen cells ± S.E.	Increase expressed as % of control	p
Control	-	305 ± 3 (57) [c]	-	-
HRBC	$5x10^8$	365 ± 4 (7)	20	.001
SRBC	$5x10^8$	381 ± 17 (6)	25	.001
CRBC	$5x10^8$	370 ± 5 (11)	21	.001
C3H [b]	$15x10^6$	400 ± 11 (5)	31	.001
Fe/FCA	250µg	383 ± 7 (8)	26	.001
EA/FCA	250µg	388 ± 7 (8)	27	.001
FIB/FCA	250µg	379 ± 4 (8)	24	.001

[a] Particulate antigens administered in 0.2 ml saline intraperitoneally; soluble protein antigens emulsified in FCA and 0.2 ml of the emulsification injected intraperitoneally.

[b] Six times frozen and thawed C3H spleen cells.

[c] Numbers in parenthesis represents the number of animals tested.

TABLE II

IN VITRO INCUBATION OF NORMAL SPLEEN CELLS WITH SIX HOUR SERUM ELICITED BY DIFFERENT ANTIGENS [a]

Six hour Serum	Ig+cells/10^3 spleen cells ± S.E.	Increase expressed as % of control	p
Control (NMS) [b]	304 ± 8 (12)	-	-
HRBC	390 ± 13 (14)	28	<.001
CRBC	369 ± 5 (5)	21	.001
FIB/FCA	383 ± 16 (4)	26	.001
Fe/FCA	384 ± 6 (3)	26	.001
EA/FCA	390 ± 5 (3)	28	.001

[a] 5×10^6 normal spleen cells incubated with 0.2 ml of 6 hour serum generated by the antigens shown or by NMS for 30 minutes at 37 °C, washed and cell concentration adjusted such that 0.8 - 1.0 x 10^6 cells in 0.1 ml medium were used for RICA assay.

[b] Normal mouse serum.

θ + lymphocytes by the cytotoxicity assay (Materials and Methods XVIII-2). Results showed a drastic decrease of 8-11% of T lymphocytes in these spleens or 28-38% of pre-immunization levels. As shown in Table III this decrease in the θ + population is statistically significant. Again, since no significant differences in total spleen cells were detected between non-immunized and immunized mice, this reduction in T cells could not be accounted for by cell migration. Conclusive evidence, however, has been provided by previous studies in our laboratory (2) showing that this decrease is due to the uptake of cytophilic γG globulin-antigen complexes by T cells. It was postulated that the acquired complexes either alters the antigenic configuration of the theta antigens or effectively blocks them from the antitheta antiserum thus resulting in an apparent reduction in the number of θ + lymphocytes.

VI. TOTAL SPLEEN CELLS IN IMMUNE ADULT BALB/C MICE

The mean number of nucleated cells in the spleens of mice 6 hours after antigenic stimulation ($1.47 \pm 0.29 \times 10^6$) did not differ significantly from that of non-immunized animals ($1.36 \pm 0.32 \times 10^6$, Results III). These results tend to support the contention that the 6 hour increase in Ig + lymphocytes were not the result of an immigration of Ig + lymphocytes to the spleen or an emigration of non Ig bearing lymphocytes out of the spleen following antigen administration.

TABLE III

PERCENT θ CARRYING LYMPHOCYTES IN SPLEENS OF MICE SIX HOURS AFTER ADMINISTRATION OF ANTIGEN

Antigen	%θ+ spleen cells ± S.E.	Decrease expressed as % of controls	p
Control	29 ± 1.0 (31)	-	-
HRBC	18 ± 0.5 (3)	38	<.001
CRBC	19 ± 0.8 (8)	34	<.001
Fe/FCA	21 ± 0.8 (4)	28	<.001
FIB/FCA	19 ± 0.6 (4)	34	<.001
EA/FCA	19 ± 1.2 (4)	34	<.001

VII. NORMAL ANTI-HRBC PFC RESPONSE

Adult Balb/c mice when immunized with 5 x 10^8 HRBC intraperitoneally, gave a potent IgM and IgG anti-HRBC PFC response. As shown in Figure 1, the IgM (19s) PFC response peaked four days after challenge while IgG (7s) responses were highest on the 6th day following immunization. Subsequent PFC assays were performed on the 4th and 6th day after immunization as a reflection of the IgM and IgG PFC response respectively, unless otherwise specified.

VIII. SUPPRESSION OF HRBC PFC RESPONSE WITH MULTIPLE INJECTIONS OF HORSE ERYTHROCYTE HEMOLYSATE SUPERNATANT (HHS)

Adult Balb/c mice were given five daily injections of 0.4 ml HHS intraperitoneally and allowed to rest for 2 days before being tested for their capacity to mount an anti-HRBC response. Results clearly showed that HHS treatment seriously compromised the ability of treated mice to respond to HRBC challenge. The degree of suppression was dose dependent and became marginally significant with two injections of HHS but reaches a maximum of about 90% with 4 injections of the tolerogen (Table IV). In all subsequent pretreatment procedures, five injections of tolerogen were given to ensure maximal suppression.

IX. SPECIFICITY OF SUPPRESSION

Unresponsiveness to HRBC was induced in Balb/c mice

Figure 1

Primary anti-HRBC response

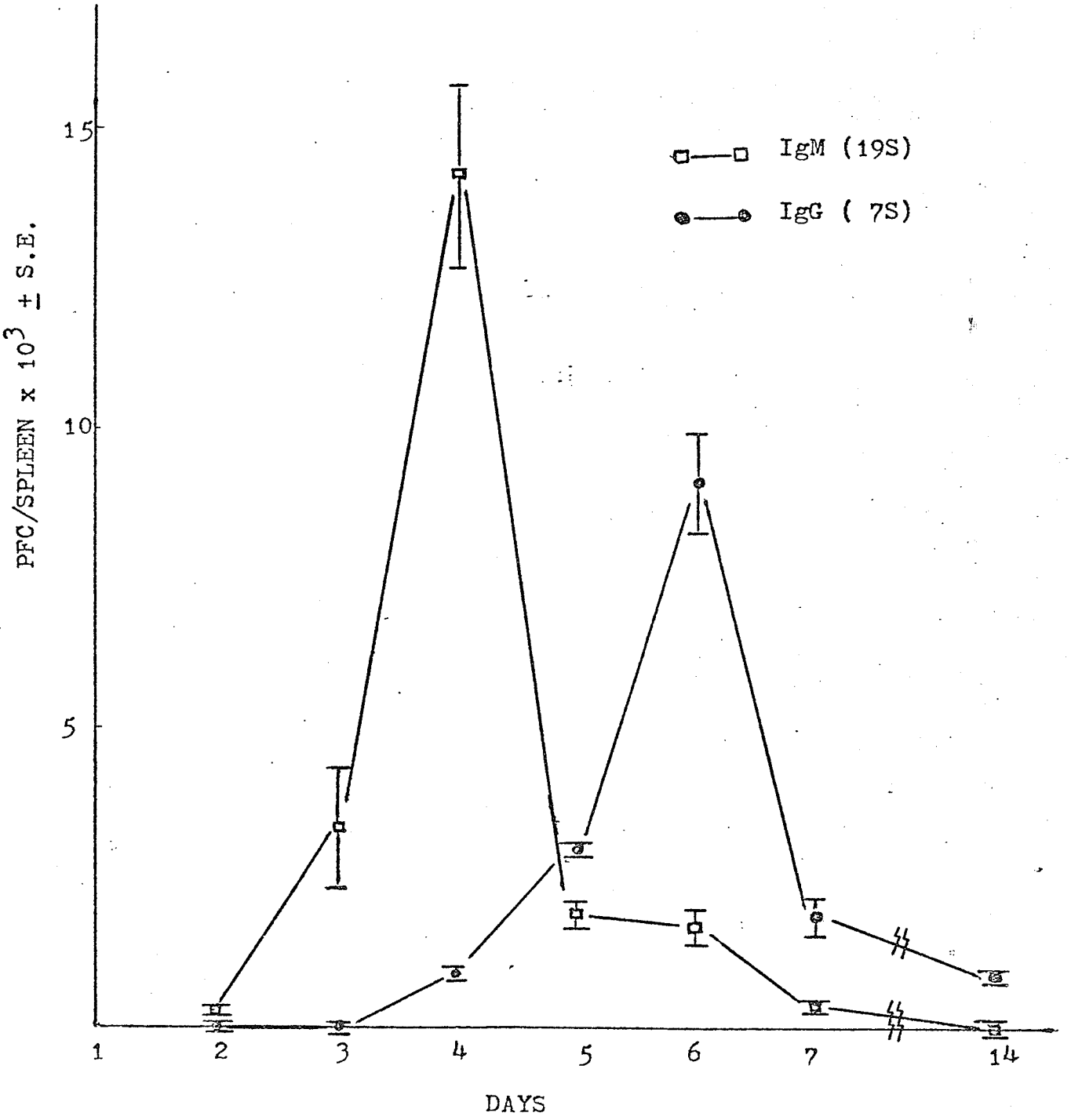


TABLE IV

INDUCTION OF UNRESPONSIVENESS TO HRBC

HHS Treatment [a]	PFC/spleen ± S.E. x 10^{-4}					
	19S	% SUP	p [b]	7S	% SUP	p
-	14.3±1.5(12)	-	-	9.2±.8(8)	-	-
1 x HHS	20.0±2.0(3)	-	N.S.	3.7±0.5(3)	60	.01
2 x HHS	7.7±0.4(3)	47	.05	5.8±1.0(3)	37	.05
3 x HHS	5.4±0.2(3)	62	.02	3.4±0.7(3)	63	.01
4 x HHS	1.7±0.1(2)	88	.01	0.5±0.03(3)	94	.001
5 x HHS	1.3±0.2(27)	91	.001	0.7±0.1(15)	93	.001

[a] Number of injections on consecutive days. HRBC ($5x10^8$) challenge always given two days after last injection of tolerogen was administered.

[b] N.S. = not significant or $p>.05$

(Materials and Methods Section XIX) and the ability of these animals to respond to heterologous antigens was tested. Table V shows the results of HRBC tolerant mice challenged with 5×10^8 SRBC or 5×10^8 CRBC two days after termination of the tolerizing regime. Experiment I in table V compared the responses of normal and tolerant Balb/c animals to CRBC challenge while the response to SRBC stimulation was shown in Experiment II. It can be seen in both experiments, that horse erythrocyte hemolysate supernatant treated mice were impaired in their ability to give a normal 19s and 7s anti HRBC response but their response to SRBC or CRBC were essentially normal. Figure 2, 3 and 4 shows the responses of normal and tolerant animals to horse spleen ferritin, egg albumin and human fibrinogen as determined by passive hemagglutination (Materials and Methods Section XXIV) technique. Both groups of animals responded equally well to these antigens. These results indicated that the treatment of mice with hemolysate abolished the humoral response to the homologous antigen but left the responses to other antigens intact.

X. DURATION OF UNRESPONSIVENESS

HRBC hemolysate supernatant treated mice were challenged with an immunogenic dose of HRBC (5×10^8) at different times after treatment to determine the duration of unresponsiveness induced. PFC assays of both IgM and IgG were performed 5 days after administration of immunogen. As seen in Table VI, treated mice when compared to nontreated control animals

TABLE V

SPECIFICITY OF HEMOLYSATE SUPERNATANT INDUCED TOLERANCE: RESPONSE OF HRBC TOLERANT ANIMALS TO SRBC AND CRBC

EXPERIMENT I

Hemolysate Treatment	Challenging Antigen	PFC per spleen ± S.E.x10^{-4} 19S	p	7S	p
-	HRBC	14.3±1.5 (12)	-	9.2±.8 (8)	-
+	HRBC	1.3±0.2 (27)	.001	0.7±.1 (15)	.001
-	CRBC	4.8±0.2 (3)	-	1.1±.07 (3)	-
+	CRBC	3.9±0.2 (3)	N.S.	1.9±0.3 (3)	N.S.

EXPERIMENT II

-	HRBC	15.3±2.4 (11)	-	17.4±4.8 (10)	-
+	HRBC	2.6±0.6 (11)	.001	1.5±0.4 (10)	.01
-	SRBC	9.7±5.0 (4)	-	6.3±1.6 (5)	-
+	SRBC	12.1±4.4 (5)	N.S.	6.0±2.8 (4)	N.S.

Figure 2

Anti-ferritin response in normal mice

and tolerant mice

X X Normal mice

⊙ ⊙ Tolerant mice

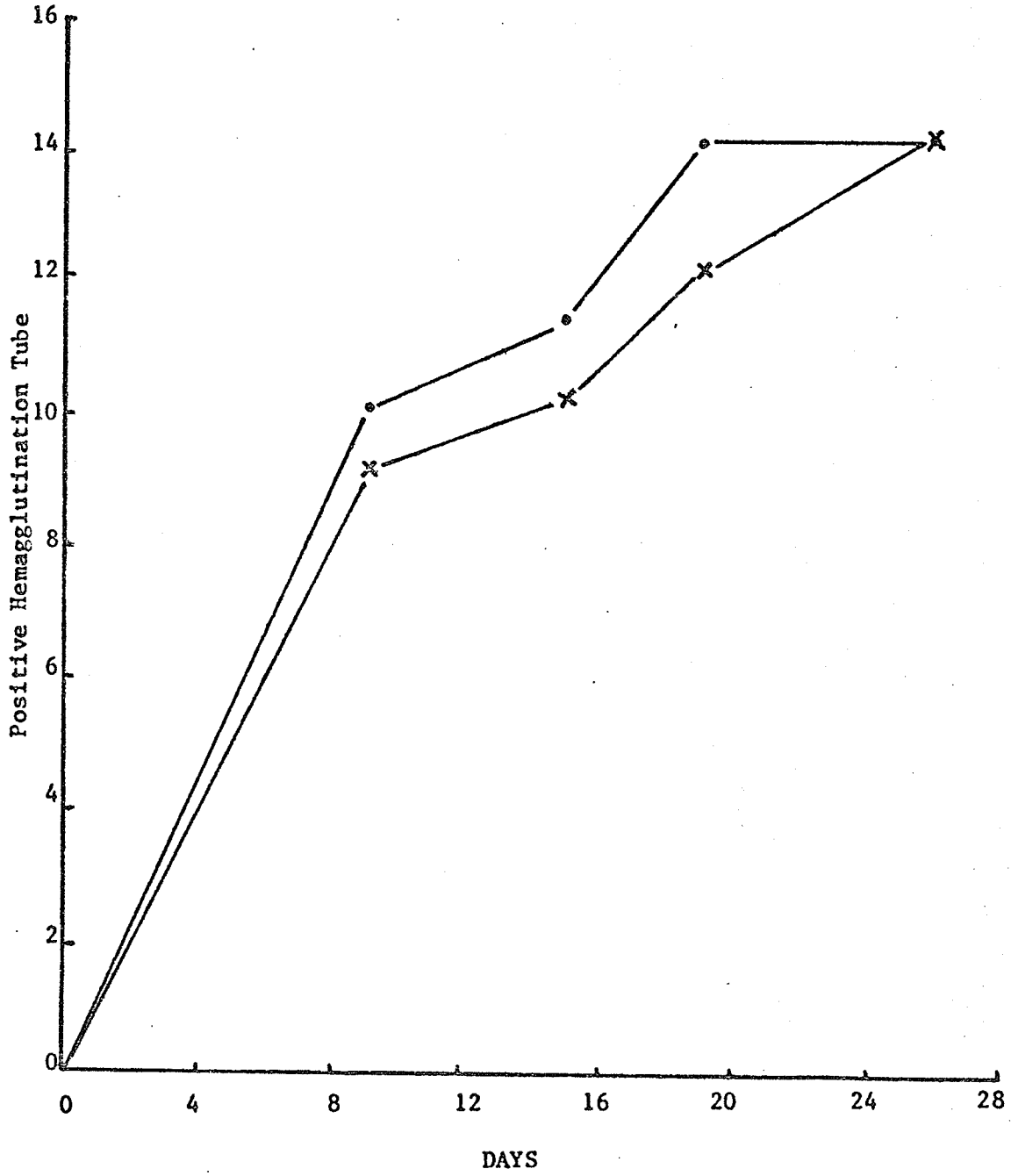


Figure 3

Anti-egg albumin response in mormal and tolerant mice.

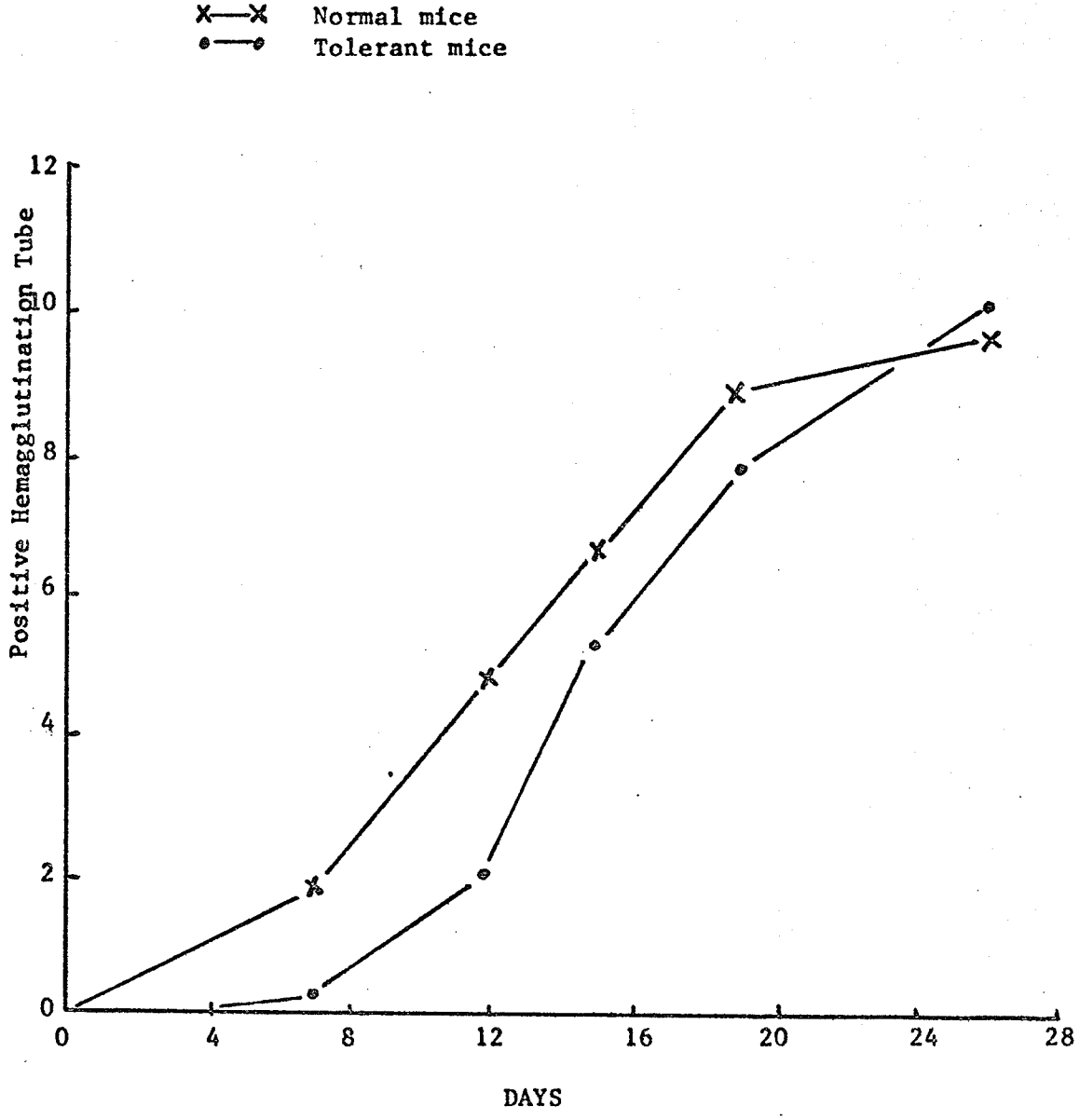


Figure 4

Anti-fibrinogen response in normal and tolerant mice

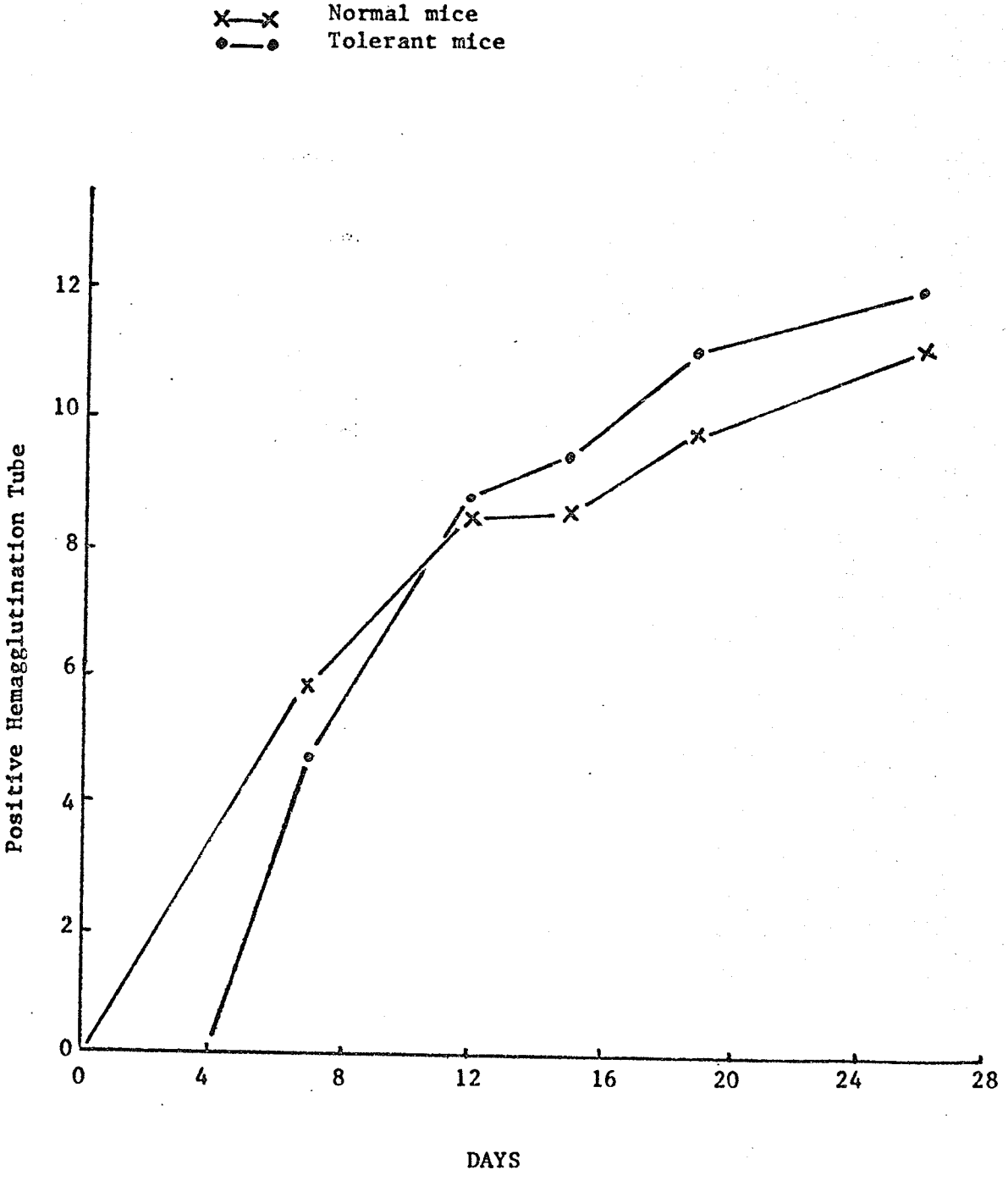


TABLE VI

DURATION OF HRBC HEMOLYSATE SUPERNATANT INDUCED UNRESPONSIVENESS

Day of immunogenic challenge [a]	Anti-HRBC PFC/spleen ± S.E. x 10^{-4} 19s Control	19s Treated	p	7s Control	7s Treated	p
2	15.3±2.4 (11)	2.6±.6 (11)	<.001	17.4±4.8 (10)	1.5±.4 (10)	<.001
21	7.1±1.0 (3)	2.7±.6 (5)	<.01	24.2±.5 (3)	15.2±2 (5)	<.02
32	7.2±2.0 (3)	0.5±.2 (3)	<.05	40.7±6.2 (4)	9.7±4.6 (3)	<.02
70	5.1±.8 (3)	1.2±.1 (4)	<.01	26.7±5.7 (3)	32.5±6.1 (4)	N.S.

a 5×10^8 HRBC given 2, 21, 32, or 70 days after last injection of hemolysate supernatant.

showed an impaired ability to mount an IgM anti HRBC response seventy days after tolerance was induced. On the other hand, although the anti HRBC IgG PFC response was still suppressed by 32 days, it was essentially normal by the seventieth day after treatment.

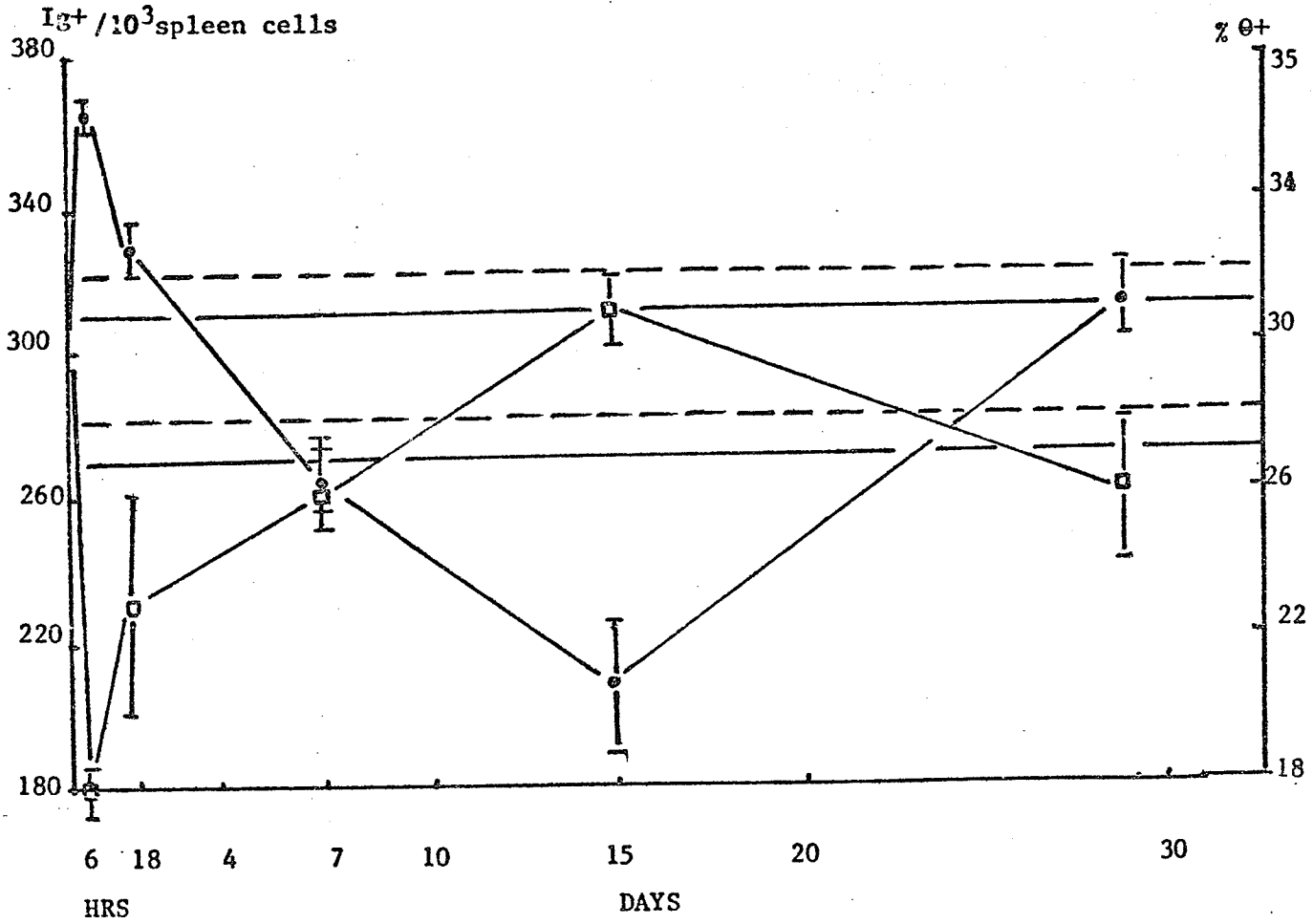
XI. CHANGES IN Ig+ AND θ+ LYMPHOCYTES IN THE SPLEENS OF NORMAL MICE DURING THE PRIMARY RESPONSE TO HRBC

The changes in Ig and θ carrying lymphocytes were followed, at various time intervals, for 29 days after an intraperitoneal injection of 5×10^8 HRBC in 0.2 ml saline. As shown in Figure 5, a statistically significant increase in Ig+ lymphocytes was observed at 6 hours after challenge. Subsequently, the number decreased with time and, after reaching a low on the 15th day, returned to pre-immunization levels by the 29th day. During this time, the level of θ carrying lymphocytes showed a marked drop at 6 hours but regained pre-immunization levels by the 7th day and remained stable at this level until the end of the experiment (Figure 5). The changes in B and T cells at 6 hours showed an inverse relationship, the magnitude of the changes being extremely similar. These findings suggest that antigen affected a large population of lymphocytes in the spleen.

XII. EFFECT OF PRETREATMENT WITH VARYING NUMBERS OF HRBC HEMOLYSATE SUPERNATANT INJECTIONS UPON THE SUBSEQUENT 6 HOUR RESPONSE TO IMMUNOGENIC HRBC STIMULATION

The ability of mice, pre-treated with one, three, or

Figure 5



Changes in Ig+ and θ+ spleen cells in normal mice following HRBC stimulation. ●—● Ig+ cells, □—□ θ+ cells; area between two horizontal dotted lines indicate range of Ig+ cells in normal spleen, area between two continuous lines indicate range of θ+ cells in normal spleen.

five injections of the tolerizing hemolysate supernatant material, to provide an increase in Ig+ lymphocytes 6 hours after challenge with HRBC was examined (Table VII). As expected, normal, non-treated animals gave a significant increase in Ig+ lymphocytes 6 hours after encounter with antigen (Group 1). In contrast, no such increase was evident in mice pre-treated with hemolysate supernate regardless of whether an immunogenic challenge was given (Group 4, 7 and 9) or not (groups 2, 3, 5, 6 and 8). In addition, pre-treated mice showed no decrease in their T cell complement 6 hours after stimulation with antigen (Groups 4, 7 and 9) as was seen with normal animals (Group 1).

XIII. CHANGES IN Ig+ AND θ+ SPLEEN CELLS IN TOLERANT MICE FOLLOWING IMMUNIZATION WITH HRBC

Tolerant mice, two days after the final injection of tolerogen, were given an immunogenic dose of HRBC (5×10^8) and spleens were removed at various time intervals thereafter to determine their content of Ig+ and θ+ lymphocytes. Results are given in Figure 6. As previously discussed (Results XII) mice pretreated with tolerogen (Table VII Group 9) lacked the increase and decrease of Ig+ lymphocytes and θ+ lymphocytes respectively seen in normal nontreated control animals stimulated with antigen 6 hours earlier. Nevertheless, like control animals, a gradual decline in Ig carrying lymphocytes occurred after 6 hours, but unlike control mice, the nadir was reached about 6 instead of 15 days after antigenic stimulation. From

TABLE VII

EFFECT OF PRE-TREATMENT WITH VARYING INJECTIONS OF HEMOLYSATE SUPERNATANT UPON THE 6 HOUR RESPONSE

Group	Hemolysate Treatment	HRBC Challenge	Ig+ per 10 spleen cells±S.E. [c]	p [d]	%θ+Spleen cells ±S.E.	p
1	no	yes	365±4 (7)		18±.5 (3)	
2	1xHHS	no	289±23 (3)	.02	30±2 (3)	.01
3	1xHHS	no	306±2 (3)	.001	31±2 (3)	.01
4	1xHHS	yes [a]	331±9 (3)	.01	29±0 (3)	.001
5	3xHHS	no	309±12 (3)	.01	32±3 (3)	.01
6	3xHHS	no	317±6 (3)	.01	29±1 (3)	.01
7	3xHHS	yes [a]	297±4 (3)	.001	29±2 (3)	.001
8	5xHHS	no	317±5 (17)	.001	30±1 (3)	.01
9	5xHHS	yes [b]	320±6 (10)	.001	27±2 (6)	.05

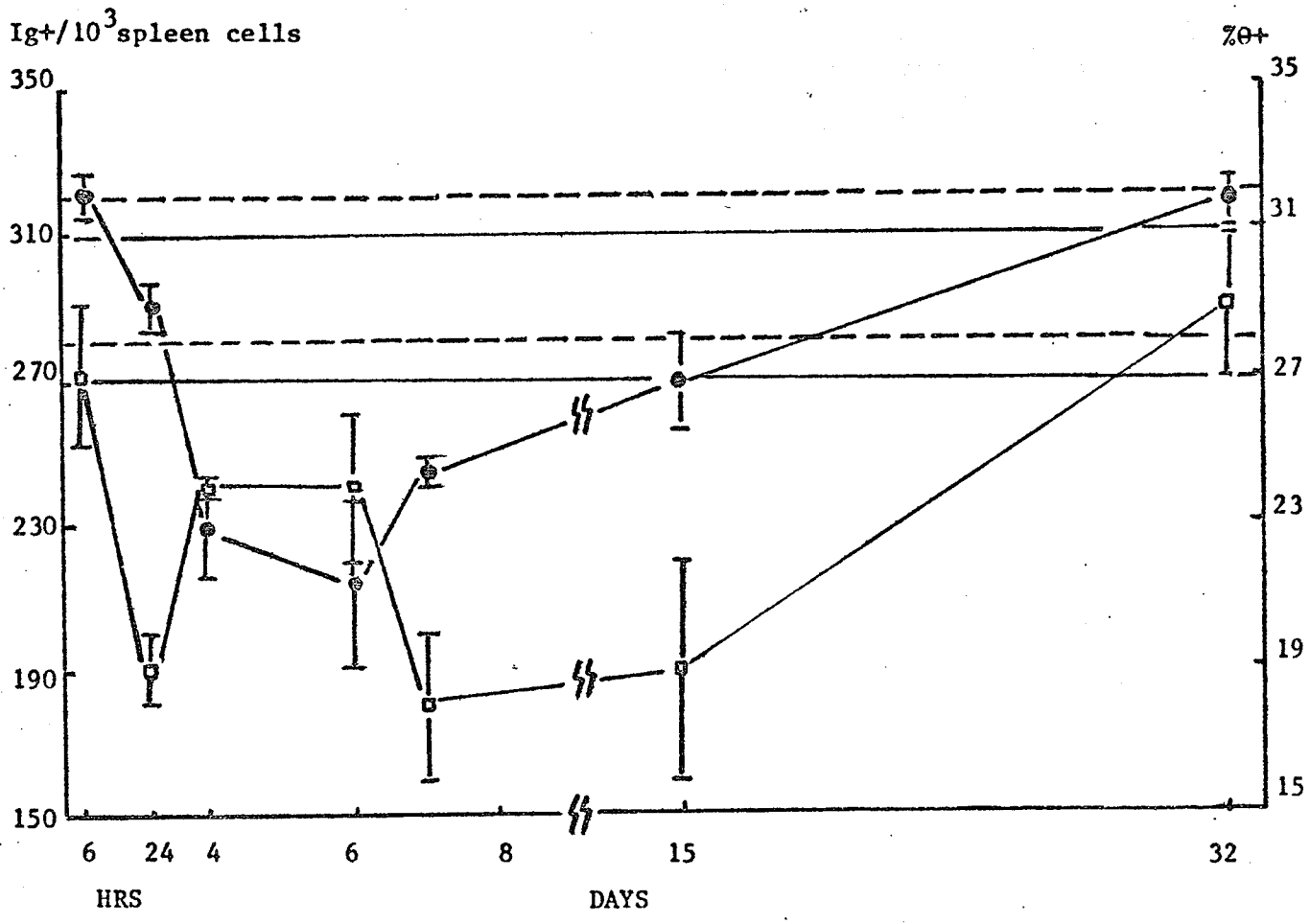
[a] HRBC challenge given 24 hours after last hemolysate injection.

[b] HRBC challenge given 48 hours after last hemolysate injection.

[c] The number of Ig+ and θ+ cells were determined at different times following the HRBC challenge or the last hemolysate injection as outlined below: -
groups 1,4,7 and 9: 6 hours after HRBC challenge;
groups 2 and 5: 6 hours after last hemolysate injection;
groups 3 and 6: 30 hours after last hemolysate injection;
groups 8: 54 hours after last hemolysate injection.

[d] Significance when compared to control (Group 1).

Figure 6



Changes in Ig+ and θ+ spleen cells in tolerant mice following HRBC stimulation.

●—● Ig+ cells, □—□ θ+ cells; area between two horizontal dotted lines indicate range of Ig+ cells in normal spleen; area between two horizontal continuous lines indicate range of θ+ cells in normal spleen.

here on the number of Ig+ cells slowly returned to preimmunization levels (e.g. by 32nd day). The changes of θ+ lymphocytes from 6 hours onwards were erratic but eventually returned to normal levels by the 32nd day. These results indirectly demonstrated a correlation between changes in surface Ig observed 6 hours subsequent to antigenic stimulation and antibody production.

In another experiment, mice treated with tolerogen were sacrificed at different time intervals after tolerogen treatment and their spleen cells examined for Ig+ cells. No significant deviation from normal, non treated levels was observed for up to 16 days after termination of tolerogen. In addition, no cytophilic complexes were observed in the serum of these animals (Table VIIIa).

XIV. DURATION OF HRBC HEMOLYSATE SUPERNATANT INDUCED INABILITY TO PRODUCE 6 HOUR RESPONSE

Mice made unresponsive to HRBC by treatment with hemolysate supernatant were examined for their ability to provide a 6 hour increase in Ig+ lymphocytes when challenged at varying times after treatment. Results indicated that treated mice were refractory to immunization for 32 days but exhibited a significant 6 hour response when tested on the seventieth day after treatment (Table VIIIb). The refractory period observed in this experiment closely resembles that observed when similarly treated mice were tested for their ability to mount humoral antibody responses (Results Section X). Taken together,

TABLE VIIIa

CHANGES IN Ig+ SPLEEN CELLS IN MICE PRETREATED WITH HEMOLYSATE SUPERNATANT*

Time after tolerogen	Ig+/10^3 Spleen cells±S.E.	
	Tolerant spleen cells	Normal spleen cells + tolerant serum
2 days	317±5(17)	304±7(10)
6 days	327±3 (3)	-**
16 days	297±5 (3)	287± (3)

* $5x10^6$ normal spleen cells were incubated with 0.2 ml serum obtained from animals sacrificed at different times after last tolerogen administration. After 30 minutes at 37°C, the cells were washed 3x with cold Hank's balanced salt solution and adjusted to an appropriate concentration for RICA assay.

** Not tested.

TABLE VIIIb

RESPONSE OF HEMOLYSATE SUPERNATANT PRETREATED MICE 6 HOURS AFTER IMMUNOGEN ADMINISTRATION GIVEN AT VARIOUS TIMES AFTER TREATMENT

Time of immunogen administration [a]	Response at 6 hours Ig+/10^3 spleen cells ± S.E.	p [b]	%θ+spleen cells ± S.E.	p
2	320±6(10)	N.S.	27±2(6)	N.S.
7	305±2(3)	N.S.	33±2(3)	N.S.
14	302±2(3)	N.S.	27±3(3)	N.S.
22	318±8(3)	N.S.	29±1(3)	N.S.
32	323±8(4)	N.S.	31±3(4)	N.S.
70	369±2(3)	<.001	23±.3(3)	<.02

a In days after last injection of hemolysate supernatant material.

b Compared with normal preimmunization levels.

the lack of 6 hour responses in tolerant animals (Results Section XII, XIII) and the reappearance of 6 hour responses, when the animals recovered from tolerance, (Results Section X, XIV) strongly suggested that the increase in Ig+ lymphocytes observed 6 hours following encounter with antigen is closely correlated with subsequent antibody production.

XV. Ig+CARRYING SPLEEN CELLS IN TOLERANT MICE SIX HOURS AFTER STIMULATION WITH HETEROLOGOUS ANTIGEN

Mice tolerized with horse erythrocyte hemolysate supernatant did not show an increase in Ig+ carrying cells 6 hours after challenge with HRBC (Results Section XII and XIII). These animals, however, were perfectly capable of responding humorally to heterologous antigen (Results Section XI). Nevertheless, when their spleens were examined six hours after challenge with various heterologous antigens, no significant increase in Ig+ lymphocytes was found (Table IX). Thus, the tolerization treatment resulted in the loss of six hour responses not only to the antigen used for tolerization, but also to other antigens even though humoral responses to these antigens are intact. These results suggests that the treatment used to induce tolerance to HRBC in some way modified the ability of treated mice to either produce or acquire cytophilic Ig or both.

TABLE IX

Ig+ SPLEEN CELLS IN TOLERANT MICE SIX HOURS AFTER HETEROLOGOUS ANTIGEN CHALLENGE

Antigen	Ig+ cells per 10^3 spleen cells ± S.E.	p [a]
-	317±5 (17)	N.S.
HRBC	320±6 (10)	N.S.
CRBC	327±15(10)	N.S.
SRBC	312±20(3)	N.S.
C3H spleen cells	287±12(3)	N.S.
BCG	292±21(3)	N.S.
Fe/FCA	297±4(3)	N.S.
EA/FCA	301±5(6)	N.S.
FIB/FCA	307±6(9)	N.S.

[a] Comparison with non-immunized normal spleen cells.

XVI. ABILITY OF TOLERANT MICE TO PRODUCE CYTOPHILIC COMPLEXES UPON STIMULATION WITH HETEROLOGOUS ANTIGENS

In order to test the possibility that spleen cells of hemolysate supernatant pretreated animals may be incapable of producing the cytophilic complexes per se, sera were obtained from treated mice 6 hours after administration of a variety of potent, cytophilic complex inducing antigens. From the data shown in Table X it was evident that a dual response was obtained based on the nature of the antigen employed. Particulate antigens were able to generate cytophilic complexes as reflected by the increase in Ig+ cells after incubation with normal spleen cells. Soluble protein antigens, however, were unable to generate cytophilic complexes since FCA 6 hour sera did not produce an increase of Ig+ cells in normal spleen cells.

XVII. UPTAKE OF CYTOPHILIC COMPLEXES BY TOLERANT SPLEEN CELLS

Sera were obtained fromnormal mice immunized 6 hours before hand with a variety of antigens. Such sera contained cytophilic complexes as reflected by significant increases in Ig+ cells when normal spleen cells were incubated in their presence in vitro (Table XI). In contrast, when spleen cells from tolerant mice were incubated with these sera in vitro, no increase in Ig+ lymphocytes were detected. These findings are in agreement with the in vivo results described in the previous section and helped to confirm the supposition of a defect existing at the cellular level rendering tolerant cells incapable of subsequent uptake of preformed cytophilic complexes.

TABLE X

ABILITY OF TOLERANT SPLEEN CELLS TO PRODUCE CYTOPHILIC COMPLEXES UPON CHALLENGE WITH HETEROLOGOUS ANTIGEN

6 hour serum [a]	In vitro incubation with normal spleen cells	
	Ig+/10^3 spleen cells ± S.E.	p [b]
- (control)	305±3 (57)	-
Saline (control)	304±7(10)	N.S.
HRBC	366±6 (11)	<.001
CRBC	368±3 (10)	<.001
C3H spleen cells	371±18(3)	<.01
BCG	377±2 (3)	<.001
Fe/FCA	301±9 (3)	N.S.
EA/FCA	302±13(6)	N.S.
FIB/FCA	303±4 (8)	N.S.
FCA	313±18(4)	N.S.

a from tolerant mice.

b compared with controls.

TABLE XI

UPTAKE OF PRE-FORMED CYTOPHILIC COMPLEXES
BY TOLERANT SPLEEN CELLS

6 hour serum from normal mice	Ig+/10^3 spleen cells ± S.E. In vitro incubation with normal spleen cells	tolerant spleen cells	p
-	305±3 (57)	317±5 (17)	N.S.
HRBC	390±13(14)	295±11(10)	<.001
CRBC	369±45 (5)	327±21 (6)	<.001
EA/FCA	390±5 (3)	307±18 (4)	<.001
FIB/FCA	383±16 (4)	319±20(11)	<.001

XVIII. EFFECT OF TOLERANT SERUM UPON UPTAKE OF CYTOPHILIC COMPLEXES BY NORMAL SPLEEN CELLS

Tolerant spleen cells were unable to acquire surface Ig presumably due to some modification of cell surface characteristics by the tolerogen. The possibility that some soluble factor(s) present in the serum was responsible for altering the surface configuration of tolerant cells was examined. Sera from tolerant mice were collected two days after termination of tolerogen treatment. Normal spleen cells were incubated with or without tolerant serum for 30 minutes at 37°C and then thoroughly washed to remove the serum. After washing the cells were further incubated for 30 minutes at 37°C in the presence of a potent 6 hour serum. At the end of the incubation period, the cells were washed and RICA assay performed. The number of Ig+ spleen cells obtained after incubation with potent 6 hour serum was 385±5 and represents a significant increase above normal values. Pre-incubation of normal spleen cells with tolerant serum before hand did not abrogate this increase (Table XII). Therefore, tolerant serum did not block the uptake of cytophilic complexes by normal spleen cells.

XIX. RESPONSE OF TOLERANT AND NORMAL SPLEEN CELLS TO PHYTOHEMAGGLUTININ STIMULATION

Phytohemagglutinin, a plant mitogen, has been shown to be a potent stimulator of DNA synthesis in peripheral T lymphocytes (396). The differential reactivity of lymphoid populations to the phytomitogen has subsequently been used as a nonspecific correlate of immunologic activity.

TABLE XII

EFFECT OF TOLERANT SERUM UPON UPTAKE OF CYTOPHILIC COMPLEXES BY NORMAL SPLEEN CELLS

Normal spleen cells	1st Incubation (Tolerant serum)	2nd Incubation (6 hour EA/FCA serum)	$Ig+/10^3$ spleen cells	p
+	-	-	314±16(5)	
+	-	+	385±5 (6)	<.001
+	+	+	374±17(6)	<.001

Spleen cells from tolerant and normal animals were stimulated with a suitable concentration of phytohemagglutinin and their responses measured by the uptake of radioactively labelled thymidine (Materials and Method XXI). Normal spleen cells incubated with phytohemagglutinin for 24-48 hours responded vigorously and incorporated 19 to 41 times more thymidine than non stimulated controls. On the other hand, tolerant spleen cells were much less reactive and incorporated only 9 to 13 times more labelled precursors compared with non stimulated controls. The mean stimulation index (See Materials and Methods XXI) given by normal spleen cells was 28±3.4 and was significantly higher than that of tolerant spleen cells (12±0.8, Table XIII). Thus, hemolysate supernatant pretreatment apparently severely affected the immunologic reactivity of peripheral T cells in the spleen.

XX. CYTOTOXIC EFFECTOR CELL FUNCTION IN TOLERANT AND NORMAL SPLEENS

Allograft immunity has been considered a manifestation of cell mediated immunity and was shown to be mediated by T cells (397-400). Specific T cells recognizing an alloantigen are stimulated in vitro or in vivo to proliferate and differentiate to form effector cytotoxic lymphocytes or killer cells. These are able to destroy target cells carrying the immunizing alloantigen, and may be assayed in vitro using the release of ^{51}Cr from labelled target cells as a measure of cytotoxic activity (401). By such assays, progenitors of cytotoxic lymphocytes were found in both mouse thymus and

TABLE XIII

PHA REACTIVITY OF SPLEEN CELLS FROM NORMAL AND TOLERANT MICE

Mean stimulation index ± S.E.		
Normal spleen cells	Tolerant spleen cells	p
28±3.4(7)	12±.8(7)	p<.001

spleen (398, 402). The ^{51}Cr release assay was used here to quantitate the cytotoxic activity of normal and hemolysate supernatant treated Balb/c spleen cells using EL-4 allogeneic tumor cells as targets. 50 x 10^6 viable spleen cells from normal or treated mice were injected intravenously via tail veins into lethally irradiated (900 R), 6-8 weeks old, allogeneic (C57 Bl/6J) recipients. After a pre-determined optimal in vivo sensitization period of 5.5 days, the recipients were sacrificed and the spleens removed. Spleen cell suspensions were prepared and the cytotoxicity assay performed as previously described (Materials and Methods XXII-2 and 3). EL-4 tumor cells, recovered from the peritoneal cavity of C57 Bl/6J mice 6-8 days after transplantation, were prepared and labelled with ^{51}Cr (Materials and Methods XXII-1) and used as target cells in the assay. Table XIV summarizes the results obtained from three separate experiments, the cytotoxic activity being reflected in the percent corrected lysis of target cells. In general, the results obtained indicated a sizeable reduction of cytotoxic activity in spleens of tolerized mice when compared to that given by normal spleens. Hemolysate supernatant treatment, therefore, affected the generation of cytotoxic effector cells or the expression of their activities.

XXI. DELAYED TYPE HYPERSENSITIVITY REACTIONS IN NORMAL AND TOLERANT MICE

Delayed type hypersensitivity represents yet another T cell mediated immunologic activity (314,403,404) and is

TABLE XIV

CYTOTOXIC EFFECTOR CELL FUNCTION IN NORMAL AND TOLERANT MICE

Expt.	Effector/target	% Corrected lysis		% Suppression
		Normal	Tolerant	
I	50	86.7(2)	36.8(2)	58
	25	90.1(2)	42.0(2)	53
II	40	90.2(4)	79.5(4)	12
III	80	57.0(3)	30.8(4)	46

characterized by delayed onset, long duration and extensive infiltration of the hypodermis and muscle with mononuclear cells. Such infiltration and the accompanying edema results in swelling of the tissue thereby affording a means of quantitating the severity of the reaction. For each antigen used, the dose required for initial sensitization and subsequently to elicit the delayed hypersensitivity reaction was characteristically different. Consequently, the optimal doses for SRBC, HRBC and ferritin were determined in preliminary experiments. For erythrocyte antigens the optimal sensitization dose was found to be 5×10^7 HRBC while that for ferritin was 250µg. The optimal testing dose was 10^8 cells for HRBC and SRBC and 125 µg for ferritin. Preliminary experiments also showed that the optimal period between sensitization and skin testing was 9-11 days for erythrocyte antigens and 3-4 weeks for ferritin. Normal and hemolysate supernatant treated mice were given intracutaneous or subcutaneous injections of either 5×10^7 HRBC, 5×10^7 SRBC or 250 µg ferritin emulsified in FCA in a total volume of 0.02 ml. After 9 days for SRBC and HRBC or 3 weeks for ferritin, the animals were skin tested for the presence of delayed type hypersentivity. Mice sensitized with erythrocyte antigens were given 10^8 cells of the homologous antigen in 0.02 ml saline intracutaneously or subcutaneously in the other foot pad while 125 µg of ferritin in 0.02 ml saline was administered in a similar fashion to mice sensitized to ferritin. Twenty to twenty four hours later, the degree of swelling of the footpads was determined by the mercury

immersion method and expressed as percent increase in footpad volume or percent swelling (Materials and Methods XXIII-2). Table XV shows composite results obtained in at least two separate experiments for each antigen. As reflected by the percent swelling in Table XV the delayed hypersensitivity reactions to HRBC obtained in ten normal animals were significantly higher than that of hemolysate supernatant treated animals (32.4±5% compared to 16.8±3, $p<.02$). Thus, HRBC tolerant mice were specifically suppressed in another T cell mediated immunologic function. Furthermore, it was found that the tolerization procedure with hemolysate supernatant also affected the ability of animals to respond to SRBC and to ferritin (Experiments II and III, Table XV). The mean swelling of footpads elicited by SRBC in normal mice was 34.5±6% but only 15.2±1% in treated mice. Similarly for ferritin, normal mice responded with a mean swelling of 26.9±2% compared to 17.1±2% in horse hemolysate supernatant treated mice.

XXII. SPECIFIC ENHANCEMENT OF ANTIBODY PRODUCTION BY SIX HOUR SPLEEN CELLS

The requirement of T helper cell function in the production of humoral responses to thymus dependent antigens (405) and to haptens on hapten-protein conjugates (406,407) has been well documented. Furthemore, spleen seeking T cells obtained 5-7 days after priming (or education) have been shown to enhance the antibody response of B cells to

TABLE XV

DELAYED TYPE HYPERSENSITIVITY TO HRBC, SRBC AND FERRITIN IN NORMAL AND TOLERANT MICE

Experiment	Antigen	Mean % swelling of footpads±S.E. Normal	Tolerant	p
I	HRBC	32.4±5(10)	16.8±3(10)	<.02
II	SRBC	34.5±6 (9)	15.2±1(10)	<.01
III	ferritin	26.9±2(13)	17.1±2 (9)	<.01

the antigen used for priming (408-412). The presence of a T cell population in the spleens of mice 6 hour after administration of antigen (6 hour T cells) has previously been demonstrated (2). However, the immunologic significance of such cells remains unknown. In this experiment, the effects of SRBC induced 6 hour T cells on antibody responses of B cells to SRBC was examined in an adoptive transfer system. Accordingly, sublethally irradiated syngeneic recipients were reconstituted with 20 x 10^6 non immune or SRBC stimulated 6 hour spleen cells and challenged with 5 x 10^8 SRBC 45 minutes after transfer of the spleen cells. Assays for both IgM and IgG anti-SRBC PFC were performed 7 days after challenge. As shown in Table XVI, the 19s (IgM) response of 6 hour spleen cells was significantly higher than that given by non immune spleen cells (6930 $\pm$1664 compared with 2664 $\pm$ 1061, $p<.05$). A similar enhancement of 7 s (IgG) PFC response was also observed (34350$\pm$8903 compared with 7109 $\pm$1567 $p<.01$). Thus, the enhancement of 19s response was only about two fold while close to a 5 fold increase in 7s PFC was obtained. The enhancement by 6 hour cells has also been recently shown to be antigen specific and to depend upon a second challenge with the priming antigen (410,411).

XXIII. EFFECT OF HRBC HEMOLYSATE SUPERNATANT PRETREATMENT ON THE ENHANCING ABILITIES OF SIX HOUR SPLEEN CELLS

The lack of 6 hour response in mice pretreated with hemolysate supernatant was described in a previous section

TABLE XVI

SPECIFIC ENHANCEMENT OF ANTIBODY PRODUCTION BY SIX HOUR SPLEEN CELLS

Normal donors		Normal recipients			
Antigen	6 hour spleen cells	Cells transferred	Antigen	PFC/spleen ± S.E. 19s	7s
(- saline)	$20x10^6$(A)	(A)	SRBC	2764±1061 (14)	7109±1567 (13)
SRBC	$20x10^6$(B)	(B)	SRBC	6930±1664 (12)	34350±8903 (12)

(Results XII, XIII). On the basis of the results obtained in Section XXII of Results, one should expect that 6 hour spleen cells from SRBC immunized, hemolysate supernatant treated mice should also lack the ability to enhance the response upon transfer to sublethally irradiated recipients. Experiments were performed to test this contention. Twenty million spleen cells from hemolysate supernatant treated mice or from similarly treated mice but additionally given SRBC for 6 hours were transferred into sublethally irradiated syngeneic recipients. Forty-five minutes after reconstitution, the recipients were challenged with 5×10^8 SRBC and PFC were determined 7 days after challenge. Results showed that spleen cells from hemolysate supernatant treated mice and those from treated mice given SRBC for 6 hours produced similar numbers of 19s antibody forming cells (2173 ± 311 and 2606 ± 281 respectively, Table XVII). These values are not significantly different from each other nor from the 19s PFC response of normal unprimed spleen cells (2764 ± 1061, Table XVI) and would seem to indicate that 6 hour cells do indeed play a regulatory role in antibody production. Similarly, the 7s anti-SRBC response of HRBC hemolysate supernatant treated and SRBC stimulated spleen cells were not significantly enhanced when compared to hemolysate supernatant treated or normal non SRBC immunized spleen cells. It should be pointed out that although no enhancement was observed for either 19s or 7s anti-SRBC response with these tolerant but SRBC primed spleen cells, they were capable of providing a normal anti-SRBC

TABLE XVII

LACK OF ENHANCING ABILITY OF 6 HOUR SPLEEN CELLS FROM TOLERANT MICE

Tolerant donors			Normal recipients		
Antigen	6 Hour spleen cells	Cells transferred	Antigen	PFC/spleen±S.E. 19s	7s
-	$20x10^6$(A)	(A)	SRBC	2173±311 (15)	20230±4371 (14)
SRBC	$20x10^6$(B)	(B)	SRBC	2606±281 (14)	10504±1157 (14)

response. An unexpected finding was the enhanced 7s anti-SRBC response given by tolerant spleen cells which have not been immunized with any antigen before transfer. The significance of this increase remains unknown at this time. These results, therefore, strongly suggests that the amplification of the immune response to SRBC, over and above the normal level, required the presence of Ig carrying T lymphocytes generated six hours after immunization with SRBC.

XXIV. PARTIAL RESTORATION OF ANTI-HRBC RESPONSIVENESS IN HRBC TOLERANT MICE BY IACF FACTOR ADMINISTRATION

Mice treated with HRBC hemolysate supernatant became unresponsive to HRBC challenge and did not give an increase in Ig carrying lymphocytes 6 hours after challenge (Results Section VIII, Table IV and Section XII, Table VII). It was also shown by previous studies in our laboratory, that a T cell soluble factor (IACF) is responsible for the cytophilicity observed in 6 hour serum. This factor was obtainable in vivo by injection of FCA or in vitro from short term cultures of BCG and thymocytes (413, 414). If the inability of hemolysate treated mice to respond to HRBC challenge is related to a deficiency in the production of this factor, it would be interesting to find out whether preformed factor could terminate the unresponsiveness. Hemolysate supernatant treated mice were divided into two groups. One group was given the factor obtained from BCG-thymocytes culture and the other given saline intraperitoneally. 45 minutes later, the animals were challenged

with 5×10^8 HRBC and PFC assays performed 4 and 6 days after challenge. As seen in Table XVIII, the anti-HRBC 19s and 7s response of control mice which did not receive any factor before challenge was $2.98 \pm .3 \times 10^{-4}$ and $2.7 \pm .2 \times 10^{-4}$ respectively. Mice given the factor gave significantly higher 19s and 7s anti-HRBC responses ($4.5 \pm .2 \times 10^{-4}$ and $4.8 \pm .1 \times 10^{-4}$ respectively). Administration of the factor did not completely terminate the unresponsive state, but significantly alleviated it.

XXV. PRESENCE OF A SUPPRESSIVE ENVIRONMENT IN TOLERANT MICE

The possibility that a suppressive environment exists in vivo in mice tolerized by repeated hemolysate supernatant treatment was investigated. Tolerant mice were sublethally irradiated two days after termination of treatment with tolerogen and reconstituted with 20×10^6 normal syngeneic spleen cells immediately following irradiation. Controls consisted of sublethally irradiated normal mice reconstituted with 20×10^6 normal syngeneic spleen cells. Sixteen to twenty four hours after reconstitution, the animals were challenged with 5×10^8 HRBC and the PFC response assayed five days later. Results showed that tolerant recipients of normal spleen cells gave a significantly reduced response when compared to the responses of normal recipients. Suppression was observed for both IgM and IgG responses (Table XIX). A suppressive environment therefore exists in the tolerant hosts.

TABLE XVIII

EFFECT OF IACF FACTOR ON ANTI-HRBC RESPONSE OF TOLERANT MICE

IACF Factor	PFC/spleen ± S.E. x 10^{-4}	
	19s	7s
-	2.98±.3(3)	2.7±.2(3)
+	4.5±.2(3) $p < .02$	4.8±.1(3) $p < .01$

TABLE XIX

PRESENCE OF A SUPPRESSIVE ENVIRONMENT IN TOLERANT MICE

Donor status	Recipient status	PFC/spleen ± S.E. 19s	7s
Normal	Normal	3950+531 (4)	7500+995 (4)
Normal	Tolerant	1283+204 (4) $p < .01$	2849+422 (4) $p < .01$

XXVI. DOES TOLERANT SERUM CONTAIN SUPPRESSIVE ACTIVITY?

Serum obtained from tolerant animals 2 days after last injection of tolerogen but without further antigenic challenge did not affect the number of Ig carrying lymphocytes in normal spleen (Table X). Its effect upon anti-HRBC PFC response of normal mice was also examined. Groups of three animals each, were given either 0.6 ml of saline or 0.6 ml of normal mouse serum or 0.6 ml of tolerant serum followed 45 minutes later by 5×10^8 HRBC. Anti-HRBC PFC were assayed 5 days after challenge. No significant differences were observed between control animals injected with saline or normal mouse serum and test animals which were given tolerant serum (Table XX). It was reasoned that the lack of suppression may be due to the masking of the suppressor activity by other soluble factors present in the serum. UM 10 filtrates of tolerant serum and normal mouse serum were therefore prepared and their in vivo effects examined. Again, no suppression was observed in mice treated with UM 10 filtrates of serum from tolerant animals, when compared with control animals treated with saline or UM 10 filtrates of normal mouse sera (Table XXI).

XXVII. LOSS OF SURFACE Ig INDUCED BY A SOLUBLE FACTOR PRESENT IN 7 DAY SERUM

Serum obtained from normal mice 7 days after immunization with antigen contained a potent Ig mobilization factor, with a mol. wt. of less than 10,000 Daltons. Incubation of normal spleen cells with these 7 day sera in vitro resulted in a marked drop in the number of Ig carrying lymphocytes (415 and

Table XXII). 7 day sera were obtained from hemolysate supernatant treated mice immunized with HRBC, CRBC and fibrinogen and tested for the presence of the surface Ig stripping factor. Results showed that such sera contained a potent factor(s) capable of stripping Ig from the surface of Ig+ lymphocytes (Table XXII). 7 day serum obtained after immunization of hemolysate supernatant treated mice with the homologous antigen, HRBC, exhibited a significantly more potent stripping activity than did 7 day HRBC immune serum from normal animals. The significance of these findings remained to be elucidated.

XXVIII. SUPPRESSIVE ACTIVITY OF 6 DAY SERUM FROM NORMAL AND HRBC TOLERANT MICE IMMUNIZED WITH HRBC

Studies in our laboratory have shown that serum collected 7 days after immunization with heterologous erythrocytes contained a factor (less than 10,000 Daltons) capable of specifically suppressing the PFC response to the antigen used for eliciting the serum (416). Since 7 day immune serum contained both suppressive activity and Ig stripping activity and since both activities are recovered in the filtrate after the serum was filtered through a Diaflo UM 10 membrane, it was conceivable that the same factor was responsible for both activities. In view of the extremely potent Ig stripping factor found in the 7 day serum of HRBC hemolysate supernatant treated mice immunized with HRBC, attention was focused on whether such sera contained suppressive activity. The experimental protocol is outlined in Figure 7. As depicted in Figure 7, sera were

TABLE XX

EFFECT OF TOLERANT SERUM UPON ANTI HRBC PFC RESPONSE OF NORMAL MICE

Treatment	PFC/spleen $\pm$ S.E. x 10^{-4}	
	19s	7s
Saline	11.3 ± 3.4 (3)	25.3 ± 3 (3)
NMS	8.0 ± 1.6 (3)	21.3 ± 1.3 (3)
Tolerant serum	10.2 ± 3 (3)	22.4 ± 1.3 (3)

TABLE XXI

EFFECT OF UM10 FILTRATE OF TOLERANT SERUM UPON ANTI HRBC PFC RESPONSE OF NORMAL MICE

Treatment	PFC/spleen ± S.E. x 10^{-4}	
	19s	7s
Saline	6.0±1.5 (4)	44.5±3.2 (4)
UM10 filtrate of NMS	6.8± .7 (4)	39.5±4.7 (4)
UM10 filtrate of tolerant sera	4.9± .6 (6)	29.0±2.6 (6)

TABLE XXII

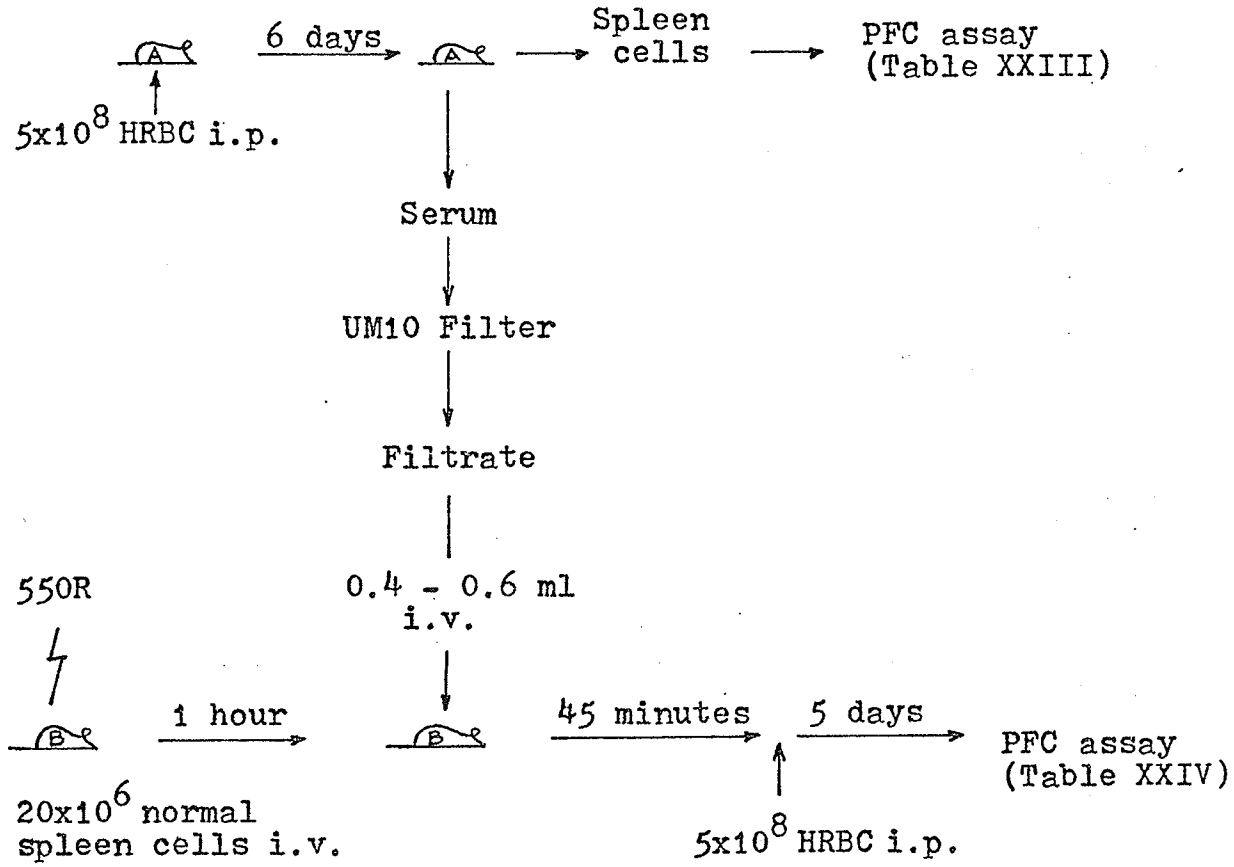
7 DAY SPLEEN CELLS AND SERUM OF NORMAL AND HEMOLYSATE SUPERNATANT TREATED MICE IMMUNIZED WITH HRBC, CRBC AND FIBRINOGEN

	Ig+/10^3 spleen cells ± S.E.					
Antigen	7 day spleen cells			7 day serum [a]		
	Normal	Tolerant	P	Normal	Tolerant	P
HRBC	246±13 (5)	246±3 (5)	N.S.	216±9 (6)	117±23 (5)	.01
CRBC	245±16 (9)	226±10 (3)	N.S.	184±8 (3)	166± 2 (3)	N.S.
FIB/FCA	224± 5 (5)	231±11 (3)	N.S.	218±7 (3)	197±10 (3)	N.S.

a Normal spleen cells incubated with 7 day serum for thirty minutes at 37° and then washed.

FIGURE 7

EXPERIMENTAL PROTOCOL: TESTING FOR SUPPRESSIVE ACTIVITY IN 6 DAY IMMUNE SERA COLLECTED FROM NORMAL AND TOLERANT MICE



Group I. Animal A: Normal Balb/c mice

II. Animal A: HRBC hemolysate supernatant pretreated mice rested for two days

III. Animal A: Same as in Group II but no HRBC was given

obtained from all three groups of animals and UM10 filtrate prepared. The immune status of donor animals from which sera were collected were also tested and results are shown in Table XXIII. As expected, tolerant mice were severely compromised in their ability to respond to HRBC challenge. As shown by the response of group III animals, tolerant mice which did not receive any immunogen exhibited only minimal antibody forming capacity. The effect of various UM10 filtrates on the subsequent response of 20×10^6 normal spleen cells were examined in an adoptive transfer system. Groups of sublethally irradiated mice were reconstituted with 20×10^6 normal spleen cells and injected with 0.4 - 0.6 ml of different UM 10 filtrates or with saline as indicated below: -

Group I UM 10 filtrate of serum from normal mice 6 days after administration of 5×10^8 HRBC

Group II UM 10 filtrate of serum from tolerant mice 6 days after administration of 5×10^8 HRBC

Group III UM 10 filtrate of serum obtained from tolerant but unchallenged mice

Group IV UM 10 filtrate of normal mouse serum

Group V Saline

Animals of all groups were challenged with 5×10^8 HRBC 3/4 hour after serum filtrates were given and PFC assays were performed on spleen cells 5 days later. Table XXIV summarizes the results obtained from two experiments. In experiment I, the UM 10 filtrates of 6 day serum from tolerant mice immunized with HRBC as well as that from non immunized tolerant mice both exhibited some suppressive activity. In experiment II, UM 10

TABLE XXIII

ANTI HRBC PFC RESPONSE OF DONOR ANIMALS FROM WHICH SERA WAS OBTAINED FOR PREPARATION OF UM10 FILTRATES

Group	Animal (A in Fig.7)	Antigen	PFC/spleen ± S.E. x 10^{-4} Experiment I 19s	Experiment I 7s	Experiment II 19s	Experiment II 7s
I	Normal	HRBC	1.5±.3 (4)	19.4±1.9 (4)	4.1±1.6 (4)	38.8±6.6 (4)
II	Tolerant	HRBC	.3±.02 (4) p <.01	1.9±.2 (4) p <.001	.9±.2 (4) p <.05	5.03±.9 (4) p <.01
III	Tolerant	-	.2±.03 (4) p <.01	.3 ±.1 (4) p <.001	-	-

TABLE XXIV

EFFECT OF UM10 FILTRATE OF 6 DAY SERUM FROM NORMAL AND HRBC HEMOLYSATE SUPERNATANT TREATED MICE IMMUNIZED WITH HRBC ON 20×10^6 NORMAL SPLEEN CELLS IN AN IRRADIATED HOST

Group	UM10 Filtrate	PFC/SPLEEN ± S.E. Experiment I		Experiment II	
		19s	7s	19s	7s
I	6 day HRBC serum from normal mice	1048+309 (4)	1649+218 (4)	269+105 (4)	747+193 (4)
II	6 day HRBC serum from tolerant mice	594+61 (3)	707+522 (3)	326+ 82 (5)	552+132 (5)
III	Serum from tolerant mice	604+123 (3)	489+ 9 0 (3)	-	-
IV	Normal mouse serum	-	-	1870+818 (3)	5117+2912 (3)
V	Saline	-	-	2909+1371 (4)	4477+1536 (4)

filtrates of 6 day immune sera from normal and tolerant animals markedly affected the response to HRBC. On the basis of these results, serum from normal and tolerant mice immunized for 6 days with HRBC as well as serum from tolerant mice not immunized with HRBC all seemed to contain some suppressive activity.

DISCUSSION

DISCUSSION

Despite significant advances in Immunology, the question of how antigen stimulates an immunocompetent cell to express its immune function still remains unanswered. There is no doubt that antigen or its processed form must first interact with the immunocompetent cell in order to initiate an immune response. That this interaction, presumably between antigen and antigen receptors on lymphoid cell surfaces, is essential but not necessarily sufficient to trigger the immunocyte is well recognized. What then is the nature of these antigen receptors on the surface of immunocompetent cells? Many investigators today favor the idea of immunoglobulin or immunoglobulin-like molecules playing the role of receptors. Such a claim is strongly supported by numerous reports in the literature of the presence of easily detectable immunoglobulin on the surface of B lymphocytes (802). Whether T lymphocytes bear surface immunoglobulin has been hotly debated over the past ten years and as yet remains controversial. Nevertheless, recent advances in this respect have made it possible to make a few generalized remarks on the subject.

In the mouse, the demonstration of receptors for immunoglobulin (Fc receptors) on normal T cells (2,3,213,220,225,227, 803) reflects the ability of these cells to bind immunoglobulin. Furthermore, some T cells that have been activated by antigen readily bind immunoglobulin (1,2,213,225,228,803-807)

either in increased amounts per cell or an increased number of cells binds Ig or both (803,808).

In humans, a small but significant percentage of peripheral blood lymphocytes forms spontaneous rosettes with sheep erythrocytes and also binds immunoglobulin (809-815).

The source of immunoglobulins found on T cells is yet another issue of controversy. Some maintain that T cell surface Ig is intrinsically synthesized (163,196,204,207) while others tend to believe that it is acquired cytophilic immunoglobulin from B cells (212,410,816,817). The general consensus at present is that some but probably not all T lymphocytes carry relatively small amounts of surface immunoglobulin which is likely cytophilic in nature.

Previous studies in our laboratory have shown that a subpopulation of splenic T lymphocytes (about 25%) acquired easily demonstrable surface immunoglobulin 6 hours following immunization (1,2). Serum collected from these animals at this time contained complexes of immunoglobulin and antigen which were shown to be cytophilic for T cells (3). Since the same proportion of T lymphocytes in the spleen has been found to be Fc receptor positive (225,227,817), it was suggested that the subpopulation of T cells in the spleen which became immunoglobulin positive 6 hours after antigenic stimulation, bind immunoglobulin or the cytophilic complexes of immunoglobulin and antigen via Fc receptors. These immunoglobulin positive T lymphocytes were subsequently referred to as 6 hour

T cells.

These data raised a number of interesting questions. What is the role of Fc receptors on T cells? What is the role of the cytophilic immunoglobulin or immunoglobulin-antigen complexes on T cells? What is the functional significance of 6 hour T cells? The solution to the first question remains controversial. While Parish (818) presented preliminary evidence suggesting that T helper cells carry Fc receptors Stout and Herzenberg (227) and Rubin and Hertel-Wulff (819) were unable to detect helper cells among Fc receptor carrying T cells. Precursors of cytotoxic T cells were also found to be Fc negative (808,820). In contrast, T cells that can amplify the cellular cytotoxic response appear to be Fc receptor positive (808) as are those T cells that respond directly to mitogen concavalin A (821,822). Cytotoxic T cells have been varyingly reported to be Fc receptor negative (823, 824), both negative and positve (825) and almost entirely Fc receptor positive (808). Evidence also existsto support the conclusion that the process of antibody dependent cellular cytotoxicity requires effector cells with Fc receptors (826-833). It is clear that antibody can have a variety of effects on the immune response. This fact, together with the observation that some of these effects appear to require an intact Fc portion of the immunoglobulin molecule, suggest that Fc receptors and therefore by inference cytophilic Ig or Ig-Ag complexes may play a role in the regulation of the immune response. Thus, antibody has been shown to interfere with the function of

cytotoxic lymphocytes and/or enhance tumor growth (790,793,834-836) and the Fc portion of the molecule appears to be important. Antibody can both inhibit and augment the humoral immune response (597). Again, an intact Fc portion of the immunoglobulin molecule was important (837-842). Antibody has also been shown to augment (616,843) but not suppress (844) the helper effect of T lymphocytes and has been implicated in suppressor T cell activity (702,845,846). The role of cytophilic complexes detected in vivo in mice at 6 hours (3) or at 5 hours in rabbits (847) is purely speculative at present. It is possible that such complexes may function as an antigen concentrating mechanism as originally proposed by Mitchison (455). Alternately, the acquisition of such complexes may activate the T cells. The data presented by Kontiainen and Mitchison demonstrating the in vitro activation of lymphocytes by T cells carrying antigen-antibody complexes are consistent with this idea (848). It should be noted that the complexes detected at 6 hours may be different from immune Ag-Ab complexes since no antibody could possibly be synthesized within 6 hours after challenge. Furthemore, evidence presented from this laboratory suggests that the formation of these complexes requires the presence of a T cell factor (413,414).

Antigen-antibody complexes have also been reported to increase DNA synthesis in lymphocytes of humans (849) although not that of mice (850). Since the 6 hour complexes are taken up by a subpopulation of T cells the next immediate question concerns the role of the 6 hour T cell . The work

presented in this thesis represents attempts to delineate its immunological significance in terms of humoral responses, cell-mediated immunity and immunoregulation. The initial working hypothesis is that if the 6 hour T cell indeed plays an important role in the immune response, it stands to reason that induction of immunological unresponsiveness would abolish its formation.

The technique employed to quantitate immunoglobulin carrying lymphocytes - the Reverse Immune Cytoadherence or RICA technique - has been well documented (98). Briefly, it involves the bridging of lymphocyte surface immunoglobulin and antigen coated erythrocytes by hybrid antibody with specificity for both immunoglobulin and the antigen. A lymphocyte with a minimum of four erythrocytes surrounding it, thus forming a rosette, (hence the term rosette forming cell or RFC) is considered to be immunoglobulin positive.

Data presented in Table I showed an increase of 20%-30% in immunoglobulin carrying lymphocytes in the spleens of adult Balb/c mice 6 hours following immunization with heterologous erythrocytes, allogeneic cells and soluble protein antigens emulsified in Freund's complete adjuvant. Concomitantly, a similar proportion of theta carrying lymphocytes in the spleen became undetectable by the antitheta serum cytotoxicity assay (Table III). Serum obtained from these animals contained complexes of immunoglobulin and antigen which is cytophilic for normal T cells in vitro (Table II). These data confirmed previous findings in our laboratory (1-3). These investigators

have also established that a factor detected in the 4S fraction of 6 hour serum from mice injected with FCA is essential for the generation of cytophilic complexes (413,414). Recently, Fridman and his associates (804,807,851-853) have also described a soluble factor, produced by educated T lymphocytes from rats or mice, which binds to immunoglobulin. It has been postulated that the surface immunoglobulin detected on 6 hour T cells represents acquired cytophilic immunoglobulin-antigen complexes which in some undefined manner (steric hindrance?) interfere with the detection of the theta antigen, thereby diminishing the number of theta positive lymphocytes as detected by the antitheta serum cytotoxicity assay. Clearly, the 6 hour T cell phenomenon represents one of the earliest detectable surface changes on T cells during a primary immune response.

The tolerance system employed in this thesis was adopted from Anderson et al (417), and is based on the finding that adult Balb/c mice could be tolerized specifically to sheep and horse erythrocytes with relative ease and without the help of cytotoxic agents. The method involves multiple injections of an ultracentrifuged supernate from hypotonically lyzed erythrocytes. The results shown in tables IV, V and VI are consistent with those of Auerbach and his associates (417,785,854) in that hemolysate supernatant treated mice were specifically tolerized and that five injections of the tolerizing material were required to attain maximal suppression which was maintained for one to two months.

Changes in immunoglobulin (B) and theta (T) carrying

lymphocytes in the spleen were observed for 30 days following an immunogenic challenge of normal Balb/c mice with 5×10^8 HRBC. The results as depicted in Fig. 5 exhibited the expected increase in immunoglobulin bearing lymphocytes and the corresponding decrease in theta carrying cells 6 hours after immunization. The immunoglobulin bearing cells subsequently dropped to a low of about 210 per thousand spleen cells by 15 days and returned to normal levels by about the 30th day. The theta carrying cells, however, returned to pre-immunization levels in approximately a week. A small molecular weight (less than 10,000 Daltons) serum factor capable of stripping surface immunoglobulin was thought to be responsible for the decrease in immunoglobulin positive cells observed (415). Recent evidence also suggests that this factor contained potent specific immunosuppressive activity (416). Its significance in tolerance induction will be discussed in a later section. Figure 6 shows the changes in immunoglobulin and theta carrying cells in HRBC tolerant animals challenged with 5×10^8 intact HRBC. The significant findings are both the lack of increase of immunoglobulin positive cells as well as the corresponding decrease in theta positive cells 6 hours following antigen challenge. These results indicate that there may indeed be some correlation between the 6 hour T cell and antibody production. It is interesting to note that tolerant mice still displayed a drop in immunoglobulin carrying cells. In view of the fact that normal 7 day immune sera contained suppressive activity (416) and the recent discovery of active

suppressor influence in tolerance induction (674,710,788), this finding may have some significance in the mechanism of tolerance and will be discussed further in a later section.

The contention that the 6 hour T cell, or the increase in immunoglobulin carrying lymphocytes in the spleen at 6 hours after antigen stimulation is essential for antibody production gained further support from a number of observations. First, a striking correspondence was observed between the recovery of the potential to mount PFC responses and the ability to exhibit 6 hour T cell response in tolerant mice (Table VI and Table VIIIb). Second, although not statistically significant, there is a tendency for mice pretreated with one injection of tolerogen to show a higher number of RFC in 6 hour spleen than those animals pretreated with three or five injections (Table VII). Both Auerbach(417) and results depicted in Table IV showed that suppression of PFC response is dependent on the frequency of tolerogen injection in the pretreatment regime. Thus animals pretreated with one single tolerogen injection were unaffected in the 19s PFC response but the 7s PFC response was only 40% of control. Those given two injections of tolerogen gave PFC responses, upon challenge with HRBC, that were only 53% and 63% of controls for 19s and 7s PFC respectively. Less than 10% of control PFC responses were obtained in animals pretreated with five injections of hemolysate supernate. Third, a T cell soluble factor (IACF) obtained from 6 hour serum of mice injected with FCA (413) or from short term cultures of BCG and thymocytes in vitro (414) has been

shown to be essential for generation of the cytophilic immunoglobulin-antigen complexes found in the serum at 6 hours. Tolerant mice treated with X_1 factor gave significantly higher PFC response when compared to non treated tolerant mice (Table XVIII) although these responses are still much lower than those obtained with non tolerant animals.

In an attempt to further substantiate the involvement of the 6 hour T cell in antibody production, it was reasoned that HRBC unresponsive animals should be capable of responding to another antigen and therefore should exhibit a 6 hour T response upon challenge with such an antigen. Table V and Figures 2, 3 and 4 illustrate the specificity of the tolerance to HRBC. Animals unresponsive to HRBC were responding normally to other erythrocyte and protein antigens. Contrary to expectations, however, no 6 hour T response were observed in any of these animals (Table IX). There are several ways to interpret these results. It is possible that the 6 hour T cell phenomenon is unrelated to antibody production per se but is involved rather in other aspects of immunity such as cell mediated immune responses. On the other hand, the 6 hour T cell may still be involved somehow in antibody formation. The data in Table IX may simply be accounted for on the basis that the tolerizing treatment may have modified the T cells in such a way that they cannot longer stabilize cytophilic complexes on their surfaces. This does not exclude an interaction of cytophilic complexes with the T cell which subsequently goes on to assist in antibody production. The third possibility

involves the 6 hour T cells in some regulatory capacity rather than the basic helper function in antibody production.

The data shown in Table XIII, XIV and XV represent results of experiments investigating various T cell mediated immunologic functions in mice made unresponsive to HRBC and which lacked 6 hour T responses.

Phytohemagglutinin, a plant mitogen, has been shown to be a potent stimulator of DNA synthesis in peripheral T lymphocytes (396). The differential reactivity of lymphoid populations to the mitogen has been subsequently used as a non specific correlate of immunologic reactivity. When spleen cells from tolerant mice were stimulated with phytohemagglutinin, DNA synthetic responses were found to be significantly suppressed as compared to normal controls (Table XIII). Allograft immunity, another well established parameter of T cell mediated immune function (397-399) was also found to be impaired in tolerant mice. Results obtained indicated a sizable reduction of cytotoxic activity in spleens of tolerant mice compared to that given by normal spleen cells (Table XIV). Finally, delayed hypersensitivity type reactivity to the specific antigen HRBC was suppressed in tolerant mice (Table XV). In addition, delayed hypersensitivity responses to heterologous antigens such as SRBC and ferritin were also suppressed. No satisfactory explanation has yet been found to account for the non-specific suppression of delayed hypersensitivity to ferritin and SRBC observed. However, a number of possibilities have been entertained. First, the unresponsiveness induced by ultracentrifuged hemolysate may in fact

be a type of non-specific suppression affecting primarily T cell mediated immunity similar to Liacopoulos' protein overloading phenomenon (504,507,855). Second, a type of antigenic competition in which only delayed type hypersensitivity is affected may be operative. Lastly, the phenomenon of 'immune deviation' (865,866) in which antigen manipulation prior to immunogenic challenge with FCA and antigen results in suppression of delayed hypersensitivity but not antibody formation offers yet another possibility. Since in our experiments the HRBC unresponsive mice responded normally to SRBC and ferritin in terms of antibody production but showed suppressed delayed hypersensity, it is entirely possible that all of the above mentioned mechanisms may be operative. Needless to say, much more work is required in order to obtain a more definitive solution.

Nevertheless, collectively, these data indicate the presence of a strong correlation between the expressions of 6 hour T cells and T cell mediated immune functions.

A number of observations lend support to the second viewpoint that the 6 hour T cell is important in the mechanism of antibody production. The most direct evidence is the lack of 6 hour T cell response in mice rendered unresponsive to HRBC as previously discussed. An apparent contradiction was the observation that HRBC tolerant mice challenged with heterologous antigens did respond normally, at least humorally, despite a lack of 6 hour T responses. To account for these observations it was postulated that the tolerization treatment

may have affected the ability of T cells to retain cytophilic complexes after interacting with them such that these T cells are activated and go on to help in antibody formation but do not carry cytophilic complexes on their surface. Consistent with such a contention are data shown in Table X and Table XI. These results clearly showed that spleen cells of tolerant mice lacked the ability to acquire cytophilic complexes (Table XI). Moreover, serum collected from these animals 6 hours after particulate antigen stimulation do indeed contain complexes cytophilic for normal T cells (Table X). However, protein antigens emulsified in FCA were unable to elicit cytophilic complexes in these tolerant mice. Reasons for such dichotomy between particulate and protein antigens in FCA are not immediately apparent. However, data from our laboratory (414) have shown that the formation of cytophilic complexes is mediated by a soluble immunoglobulin antigen complexing factor (IACF). As reported, IACF was inducible in vivo by particulate antigens and soluble protein in FCA (413) as well as in vitro by mycobacteria, SRBC, allogeneic cells and soluble substances such as LPS or polyadenylic-polyuridylic acids known to be potent adjuvants (414). Thus it seems that the ability of a substance to generate IACF, and therefore cytophilic complexes, reflects its adjuvanticity. Although these findings showed that substances with what Dresser (856) considers as intrinsic adjuvanticity (e.g. SRBC and allogeneic cells) or extrinsic adjuvanticity (e.g. mycobacterium, LPS and polyA-U) are all capable of generating IACF, it is quite possible that different

inductive mechanisms exist. Such a difference may have been uncovered by the hemolysate treatment which affects one mechanism of IACF induction and not the other. The possibility of a different time sequence of generating cytophilic complexes in response to serum protein FCA in tolerant mice cannot be discarded.

Evidence supporting the regulatory function of the 6 hour T cell was initiated by other investigators in our laboratory and confirmed by work presented in this thesis. Thus, it was shown that spleen cells collected 6 hours after priming with SRBC, markedly augmented the anti-SRBC PFC responses in sublethally irradiated recipients. The 19s PFC response more than doubled as compared to that obtained wiht non primed spleen cells while the 7s PFC response was almost five times as great(Table XVI). This enhancement was antigen specific (410). No such amplification was observed when 6 hour SRBC educated spleen cells from HRBC tolerant mice were compared with spleen cells from HRBC tolerant but non SRBC stimulated animals (Table XVII). These findings, together with the fact that 6 hour SRBC educated spleen cells from HRBC tolerant mice lacked 6 hour T cells (Table IX) strongly implicates the 6 hour T cell as having a regulatory role in antibody formation. In addition, spleen cells from tolerant mice with or without prior priming with SRBC, gave upon challenge with SRBC, responses similar to those given by normal unprimed spleen cells (Table XVI & XVII). This led us to conclude that the 6 hour T cell represents a regulatory mechanism and

acts to amplify the basic helper cell function whatever this basic helper cell function may be. Recently, studies in our laboratory provided conclusive evidence that the specific enhancement of antibody response by 6 hour T cells is the result of a synergistic interaction between an Ig+ and an Ig- but θ+ subpopulation of primed T cells (411). The Ig+ cell presumably has antigen on their surface.

The idea that two T cells may collaborate in providing helper cell function finds support in the work of Janeway on the 'premium effect' (857). However, he was unable to isolate these subpopulations on the basis of their susceptibility to X-irradiation (858) anti θ serum, anti thymocyte serum and effect of thymectomy (495). Kappler et al (493), on the other hand, were able to distinguish two subpopulations of primed T cells involved in helper cell function, on the basis of their dependence on the presence of the thymus or their sensitivity to anti thymocyte sera (494). As well, enhancement of helper cell function via passively acquired antibody by primed T cells has also been demonstrated in mice (616) and in chickens (624, 859), although in our case the surface Ig is not likely to be antibody.

In summary then, on the basis of the data presented in this thesis and those from other studies done in our laboratory, we would like to entertain the idea that as early as 6 hours following administration of antigen, a subpopulation of T cells in the spleen (about 25% and likely the same subpopulation which carries Fc receptors) acquires cytophilic complexes

of Ig and antigen, thus becoming Ig positive and theta negative, due possibly to steric hindrance. This subpopulation of Ig+ T cells (the 6 hour T cell) subsequently co-operates synergistically with the remaining Ig negative and θ positive T cells resulting in a markedly augmented antibody response. The Ig+ T cell is believed to act as a regulator of helper cells for antibody production and may be distinct from the conventional helper T cells. The 6 hour T cell's regulatory capacity may also extend beyond antibody production and may encompass other cell mediated immune reactions as suggested by work in this thesis. Such regulator T cells have been shown to exist for other T cell functions (516,665,860).

The mechanisms involved in the ability of an individual's immune system to distinguish between its own and foreign antigens have been one of the most important areas of immunological investigation. The finding that, under certain experimental conditions, specific unresponsiveness could be elicited towards foreign antigens has greatly facilitated our understanding of the mechanisms responsible for self-tolerance. It is clear that immunological tolerance is an exceedingly complex state with many contributing mechanisms that are not necessarily mutually exclusive (861). Mechanisms implicated in the development of a tolerant state include clonal deletion (4) or abortion (862), blockade of lymphocyte antigen receptor by antigen-antibody complexes (774,854) or by antibodies to surface receptors (863,864). Recently, it has become clear from studies of a number of laboratories (710) that an active

process of T cell-mediated suppression also plays a role in the development of immunological tolerance. Studies in our laboratory have established the existence of a small molecular weight suppressive factor in the serum of mice immunized for seven days (416). Such a factor is also capable of causing a decrease in the number of immunoglobulin carrying lymphocytes in a normal spleen population in vitro (415) and is thought to be responsible for the decrease in immunoglobulin carrying lymphocytes observed in vivo between 6 hours and 15 days during aprimary response (1 and Figure 5). A similar decrease in immunoglobulin carrying lymphocytes was observed in tolerant mice despite the absence of an increase at 6 hours following antigen challenge (Figure 6). In addition, serum collected 7 days after immunization of tolerant mice contained potent surface immunoglobulin stripping activity (Table XXII). These data suggest a possible involvement of an active suppressor mechanism in our tolerance system. Experiments were subsequently carried out to investigate this contention. The presence of a suppressive environment in tolerant animals is evident from the results depicted in Table XIX. Normal syngeneic spleen cells transferred into tolerant recipients were significantly suppressed in their ability to respond to HRBC challenge when compared to their response in normal hosts. A number of workers have also observed that the adoptive transfer of normal, syngeneic immunocompetent cells or even immunized cells into tolerant animals does not abrogate certain tolerant states (642,650). Further studies involving the

transfer of tolerant serum or their UM10 filtrate into normal syngeneic animals failed to show any suppressive effect on subsequent anti HRBC PFC response (Table XX & XXI). It would seem that serum of tolerant animals does not contain any suppressive activity. Nevertheless, it is entirely possible that the lack of suppressive activity is a matter of quantity and/or route of injection. Also, the generation of suppressive activity may require an additional antigenic challenge in vivo as is the case in previous observations. Additional experiments involving in vivo and/or in vitro preincubation of smaller numbers of normal spleen cells with tolerant serum prior to testing in irradiated hosts may serve to elucidate this problem.

Since 7 day serum collected from tolerant mice immunized with HRBC contained potent Ig stripping activity (Table XXII), their effect on anti HRBC PFC response of normal spleen cells was assayed in an adoptive transfer system. Table XXIV represents the results of two such attempts. It is clear that both 6 day HRBC immune serum from normal and tolerant mice contains suppressive activity, although more experiments will have to be done before any definitive statements could be made regarding the differences in their suppressive potency. An interesting finding is that of the response of 20×10^6 normal spleen cells following treatment with UM10 filtrate of tolerant serum (Group III, expt. I). In contrast to the apparent lack of effect of whole tolerant serum (Table XX) upon the anti HRBC response in normal mice, the

diminished response obtained with $20x10^6$ normal cells suggests that tolerant serum may indeed contain potent suppressive activity which becomes evident only under more appropriate conditions. Despite these suggestive findings, it must be pointed out that these are merely preliminary data and are far from being conclusive. Other mechanistic possibilites definitely exist , one of which is Auerbach's (854) recent proposal of 'Allosteric tolerance' mediated through tolerogenic determinants present in the hemolysate supernatant material. It is quite possible and probable that more than one non mutually exclusive mechanism may account for the hemolysate supernatant induced tolerance to heterologous red cells.

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