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COMPARISON OF THE EASE OF TOLERANCE INDUCTION IN THYMUS AND BONE MARROW OF THREE STRAINS OF MICE

A DISSERTATION

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COMPARISON OF THE EASE OF TOLERANCE INDUCTION IN THYMUS AND BONE MARROW OF THREE STRAINS OF MICE

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CHAPTER I

INTRODUCTION

In the early days of immunology, scientists recognized the necessity for a mechanism whereby the body can recognize "self" antigens and thus fail to mount an immunologic response to those body constituents. Indeed, in his early theory of antibody formation set forth in 1901, Ehrlich (Ehrlich and Morgenroth, 1957) proposed a method for such self-recognition.

Owen (1945) observed that dizygotic cattle twins, sharing the same placental circulation, possessed red cells of their own blood group as well as those of their twin. Based on this and other observations, Burnet and Fenner (1949) proposed that there was a period of time during embryonic development in which contact with immunogenic materials resulted in recognition of self, whereas contact after this period of maturation elicited an immune response. Soon, Billingham, Brent and Medawar (1953) observed that if bone marrow or spleen cells were transferred from one strain of mice to another at the time of birth, the recipient mice would not reject skin grafts from the donor strain when grafted later in life. The immune anergy produced was specific, since

these animals did reject skin from a third strain of mice. The condition in which an animal has lost its ability to respond to a specific antigen was called immunologic tolerance. This state is also often referred to as immunologic paralysis or immunologic unresponsiveness.

Initially, it was believed that tolerance could only be produced in the neonatal period with living, replicating cells. However, Felton and Ottinger (1942) had produced unresponsiveness with pneumococcal polysaccharide in mice, and Hanan and Oyama (1954) reported tolerance induction with a soluble protein antigen. Since these early investigations, tolerance has been produced in neonatal and adult animals to a variety of soluble antigens (Smith, 1961; Dresser and Mitchison, 1968).

Although immunity and tolerance may appear to be opposing events, they share at least one common feature; that is, both result from the interaction of antigen with immunologically competent cells. For this reason it is necessary to know the sequence of cellular events which occur during the production of an immune response before one can understand the specific cellular "lesion" in immunologic unresponsiveness.

Cells Involved in the Immune Response

In recent years it has become apparent that several cell types may participate in the production of a humoral immune response. Any attempt to completely review all of the literature on this subject would be impossible. However, the reader is referred to several excellent reviews by Abdou and Richter (1970a), Talmage, Radovich and Hemmingsen (1970), Playfair (1971b), Miller, Basten, Sprent, and Cheers (1971), Schwartz, Ryder and Gottlieb (1970), Silverman (1970) and Weigle,

Chiller, and Habicht (1972). Both in vivo and in vitro studies have identified the cellular components which may cooperate in order for antibody production to occur. Three cells have been identified thus far. They include the macrophage and two types of lymphoid cells.

The information covered in this discussion will address itself only to the cellular events involved in the production of a humoral antibody response and will not attempt to discuss the multitude of cellular interactions which are involved in cell-mediated immunity.

The Macrophage

As early as 1882, Elie Metchnikoff observed the phenomenon of phagocytosis (Metchnikoff, 1907) and proposed that phagocytic cells played an essential role in host resistance to infection. The concept of the reticuloendothelial system (RES) was conceived by Aschoff (1924). His system was based on the ability of the RES to ingest vital dyes and other particulate material. Thus, the RES was composed of all of the phagocytic cells of the body.

Several lines of investigation have shown that macrophages may participate in eliciting an immune response in animals challenged with immunogens. Among these are studies utilizing blockade of RES function, macrophage transfer into intact animals, and macrophage function during in vitro immune responses.

Sabet, Newlin and Friedman (1968) and Sabet and Friedman (1969b) studied the effect of blockade by colloidal carbon of the RES on the formation of anti-sheep erythrocyte antibody. If carbon was administered 24-48 hours before antigenic challenge, there was marked suppression of the ability of spleen cells to make antibody to sheep red blood cells

(sRBC). Treatment of animals with carbon on the same day that antigen was administered was less effective in suppressing the immune response, while treatment with carbon 1-2 days after immunization had little effect on the plaque forming cell response. Sabet and Friedman (1969a) reported that RES blockade also interfered with the production of a normal secondary response to sRBC in primed mice. Carbon pretreatment was most effective in suppressing the secondary response if given 1-2 days prior to antigenic challenge. In addition, it was discovered (Sabet and Friedman, 1969b) that mice injected with carbon before primary stimulation with sRBC had normal direct (19S) plaque forming responses after a second antigen injection given one month later. However, these animals failed to respond with an indirect (7S) PFC response. Sabet and Friedman called this phenomenon specific immunologic "amnesia" in contrast to specific immunological tolerance as the carbon treated mice responded to secondary challenge with antibody production similar to that seen in primary immunization of normal mice. Since further studies (Friedman and Sabet, 1970) revealed that RES blockade with carbon had only a moderate effect on the number of stem cells capable of differentiating into antibody forming cells, the effects described above could not be attributed to direct action of carbon on the precursor of the antibody producing cell.

Other reagents which either destroy macrophages or inhibit phagocytosis have likewise been found to be immunosuppresive (Panjel and Cayeux, 1968; Jehn, Musher and Weinstein, 1970; Bice, Gruwell and Salvaggio, 1972). Antiserum prepared against macrophages was reported by Panjel and Cayeux (1968) to suppress antibody production <u>in vitro</u>, while

Jehn et al. (1970) observed that anti-macrophage serum inhibited the ability of specific antigens to elicit in vitro blast transformation of lymphocytes from sensitized guinea pigs. Anti-macrophage treatment did not affect blast transformation by phytohemagglutinin (PHA). Carrageenan, a macrophage toxic substance, when given intraperitoneally resulted in complete suppression of an immune response to a subsequent intraperitoneal injection of sheep erythrocytes (Lake, Bice, Schwartz, and Salvaggio, 1971; Bice, Gruwell, and Salvaggio, 1972). If the red blood cells were administered intravenously there was no reduction of the PFC response to sRBC. These authors assumed that carrageenan inhibited the ability of peritoneal macrophages to process and/or transport antigen from the peritoneal cavity. During in vitro cultures carrageenan was immunosuppressive if given prior to or up to 6 hrs after antigen. No suppressive effect occurred if carrageenan was added 17 hrs after antigen. Others (Ascheim and Raffel, 1972) reported that the immunosuppressive activity of carrageenan was due to its action on lymphoid cells and that this substance did not inhibit macrophage activity.

In 1966, Martin found that the inability of newborn rabbits to respond immunologically to bovine serum albumin (BSA) could be attributed to a deficiency in the macrophage activity of the neonate. Newborn rabbits treated with BSA plus adult macrophages could mount an antibody response, whereas neonates which were not given adult macrophages were unresponsive to this antigen. A similar observation was reported by Braun and Lasky (1967) who observed that adult peritoneal cells which adhere to glass allow newborn C57BL mice to respond to sRBC. Normally these animals fail to respond to this antigen until 5-10 days after

birth. The antibody produced by the recipient mice was made by cells of the recipient, since irradiation of recipients prior to transfer of adherent cells abolished the response. These results were confirmed by Argyris (1968) who reported, in addition, that administration of adult macrophages to C3H mice before or after a three day period resulted in no immune response to sRBC. Argyris noted that a critical number of macrophages was required to enhance the anti-sRBC response of the newborn mouse and that high doses of macrophages were ineffective.

Gallily and Feldman (1967) and Feldman and Gallily (1967) presented evidence that two cells were required for an immune response to Shigella paradysenteriae. Peritoneal macrophages which had been incubated in vitro with shigella antigen and injected into sublethally irradiated recipients elicited an agglutinating antibody response to shigella. Irradiated animals were unable to respond to injection of the antigen alone. Because lymph node cells preincubated with antigen did not produce this effect and because lymphocyte-free macrophages did, these authors excluded the possibility that the response observed in the recipient was produced by lymphocytes which might have been present in the donor peritoneal cell inoculum. If recipients were given a higher, lethal irradiation dose, no antibody production resulted from injection of antigen treated macrophages. Additional support for a two cell requirement came from experiments which showed that lethally irradiated mice only produced a significant titer of agglutinating antibodies if they were reconstituted with normal lymphocytes and antigen-treated macrophages. If macrophages were taken from irradiated donors they were unable to evoke an immune response in these cell-transfer experiments.

Because irradiation did not inhibit the ability of macrophages to phagocytize sRBC, it was postulated that irradiation had interfered with some step of antigen processing within the macrophage.

Gershon and Feldman (1968) confirmed the work of Gallily and Feldman using Shigella paradysenteriae; however, when they tried to substitute an erythrocyte antigen, they were unable to reconstitute sublethally irradiated recipients with antigen-treated macrophages. Because these authors had observed that thymus explants added to spleen organ cultures of irradiated animals previously inoculated with macrophages and antigen in vitro would cause a resultant antibody response, they pointed out that the sRBC response may require a third cell type which could be supplied by the thymus.

Transfer of sRBC containing macrophages (Argyris, 1967) or macrophages containing formalin-killed pneumococci or pneumococcal capsular polysaccharide (Argyris and Askonas, 1968) to normal syngeneic recipients was followed by an immune response to those antigens. No antibody was produced in recipients which had been lethally irradiated, suggesting that cells of the irradiated host were the actual antibody producers.

Pribnow and Silverman (1967) found that rabbit peritoneal macrophages (PEC), when sensitized in vitro to bovine gamma globulin (BGG), would provoke an antibody response to that antigen when transferred to normal recipients. If the antigen-sensitized peritoneal cells were given to irradiated recipients, no antibody was produced unless normal lymphoid cells were transferred at the same time as peritoneal cells. The function of the peritoneal cell was sensitive to irradiation, as PEC

from irradiated donors treated *in vitro* with antigen were ineffective in combination with normal lymphoid cells in evoking an immune response to BGG. The authors suggested, therefore, that inhibition of macrophage function was the initial radiosensitive phase of the immune response.

Using *Maia squinado* hemocyanin (MSH) as antigen, Unanue and Askonas (1968a) found that antigen associated with macrophages was more effective in priming animals for a subsequent secondary response than was free antigen. Macrophage-associated MSH produced a secondary response in irradiated recipients provided that normal lymphoid cells were also administered. Irradiation of the macrophage donors, however, did not result in a lowering of the immunogenicity of the macrophage-associated hemocyanin.

Several protein antigens (BSA, human serum albumin, lysozyme and ovalbumin) were compared for their immunogenicity in free form or associated with macrophages by Mitchison (1969a). These studies showed that macrophages enhanced the immunogenic properties of these protein antigens and that irradiation of peritoneal cell donors before antigen administration abolished the enhancing properties of the macrophage.

It is apparent from the above reports that studies of the radiationsensitivity of macrophages are conflicting (Mitchison, 1969a; Unanue and Askonas, 1968a; Roseman, 1969; Pribnow and Silverman, 1967). Histologic studies (Brecher, Endicott, Gump and Brawner, 1948) and experiments showing that whole body irradiation does not influence removal of particulate material from the blood stream (Benacerraf *et al*. 1959; DiLuzio, 1955) indicated that this cell was relatively resistant to irradiation. However, in other experiments irradiation of macrophages

altered their bactericidal activity and their ability to digest foreign red blood cells (Donaldson et al. 1956). Kolisch and Mitchison (1968) reported that irradiated macrophages ingested BSA as well as normal cells but that the irradiated cells did not retain antigen as well as their normal counterparts. All of these facts suggested that macrophages may develop what has been called "radiation indigestion" (Schwartz, Ryder, and Gottlieb, 1970). Differences in the sensitivity of macrophages to irradiation may also reflect differences in the handling of the various antigens used in these studies. It is possible that macrophages play no role in the immune response to some antigens, or that the immunogenic function of macrophages with regard to different antigens varies. For example, hemocyanin may not require the radiosensitive function (Unanue, 1969) which is necessary for macrophages to render protein antigens immunogenic (Kolisch and Mitchison, 1968).

Frisch and Wilson (1969) and Many and Schwartz (1970) discovered that mice which were suppressed with cyclophosphamide did not respond to sRBC, whether or not that antigen was associated with macrophages. Macrophages of cyclophosphamide-treated donors could ingest erythrocytes and trigger antibody synthesis to the red cell when injected into normal mice, therefore cyclophosphamide did not suppress the function of the macrophage. On the other hand, cycloleucine treated mice could respond with antibody production when challenged with antigen associated with normal macrophages (Frisch and Wilson, 1969). Macrophages from cycloleucine treated animals were immunologically inert, even though they could phagocytize antigen as well as normal macrophages. Such experiments suggested that at least two cells were required for an immune

response to erythrocytes, and that engulfment of antigen *per se* by macrophages did not insure that macrophage function was intact.

Mishell and Dutton (1966, 1967) developed an *in vitro* system for immunizing spleen cells with erythrocyte antigens. Their procedure involved the culture of dissociated spleen cells with foreign erythrocytes for four days. At the end of the incubation period spleen cells producing antibody were enumerated by the Jerne plaque assay for direct plaque forming cells (PFC) to the red cell antigen. This technique has allowed these and other investigators to analyze exactly what cell types are required for the *in vitro* immune response.

At least two cell types were found by Mosier (1967) to be necessary for the *in vitro* response of spleen cells to erythrocyte antigens. These cells were separated according to their ability to adhere to glass. Adherent and non-adherent cells alone were unable to respond to antigenic stimulation, but if they were put together in culture with antigen an immune response ensued. Mosier reported that incubation of adherent cells with antigen for 30 min, followed by thorough washing of the cells, endowed these cells with the ability to induce antibody production to sRBC in the non-adherent cell population. This work was extended by Mosier and Coppelson (1968) who cultured serial dilutions of one cell population in the presence of a constant, large number of the other population of cells. The log of the PFC response was plotted against the log of the diluted cell population present in the culture. When adherent cells were diluted the slope of the regression line was 1.0, whereas dilution of the non-adherent population gave a regression line with a slope of 1.9. When diluted, unseparated spleen cells were

used the slope of the line was 2.6. Mosier and Coppelson concluded from these data that three cells were involved in the in vitro immune response: an adherent cell and two non-adherent cells. Mosier (1969) found, in addition, that spleen cells formed clusters while undergoing an immune response to sRBC in vitro. These clusters contained most of the antibody producing cells in the culture and disruption of the clusters inhibited the appearance of PFC in the cultures. Specific reaggregation of clusters occurred, which could be specifically inhibited by adding antibody to sRBC to the culture. Addition of two types of erythrocytes (sheep and burro) to the cultures resulted in individual clusters producing antibody to only one of the two RBC types. Many of the cell clusters contained monocytes as well as lymphocytes.

Pierce and Benacerraf (1969) and Pierce (1969, 1970) confirmed Mosier's discovery that cell clusters were required for in vitro antibody production. In their experiments clusters appeared within six hrs of the beginning of culture if the cultures were incubated on a rocking platform, and the clusters remained for the duration of the incubation period (4 days). If cultures were incubated without rocking, fewer clusters and fewer PFC resulted. Adherent, phagocytic cells were found to be responsible for cluster formation. Anti-sRBC antibody specifically suppressed the PFC response against sheep erythrocytes if added at the beginning of culture or 24 hrs later, indicating that during this 24 hr time period the culture system was antigen dependent. Various other alterations in the culture conditions allowed the identification of two steps necessary for the in vitro antibody response. The first step depended upon the participation of macrophages and cluster formation, and

required 24 hrs to go to completion. The second step did not depend on presence of macrophages. It, too, was of 24 hrs duration and probably signaled the initiation of cell transformation in the non-adherent lymphocyte population. After the first 48 hrs activated lymphocytes could develop into plaque forming cells independently, without antigen, macrophages or cell clusters.

Hartman and coworkers (1970) and Mishell, Dutton and Raidt (1970) found that spleen cell populations could be separated on discontinuous albumin gradients due to differences in their density. By use of this technique, they obtained four bands of cells which they called A, B, C, and D. When cells from band A or D were cultured alone with sheep red blood cells, no antibody production occurred. However, small numbers of cells from the A band added to the D band cultures allowed an anti-erythrocyte response. They further analyzed the in vitro antibody response in regard to the requirement for adherent and non-adherent cells. As had been reported by Mosier, they found that both cell types were required. Utilization of anti-H-2 serum and donors who differed at the H-2 histocompatability locus allowed the identification of the precursor of the antibody forming cell as a member of the non-adherent spleen cell population. It was reported by Dutton, McCarthy, Mishell and Raidt (1970) that A band and adherent cells were equally effective in restoring the response of D band cells, and that cell free supernates of these cells could also restore the ability of non-adherent or D band lymphocytes to respond to antigenic stimulations. This was true whether or not the cells were preincubated with antigen. The phenomenon could not be attributed positively to macrophage function, since A band cells were markedly

depleted of macrophages. Hoffman and Dutton (1971) found that the factor in supernates of macrophages could be absorbed by red cell antigens but that the absorption was not antigen specific. Additionally, they observed that treatment of erythrocyte antigens with macrophage supernates rendered these cells capable of stimulating non-adherent cells in the absence of attached cells or attached cell supernates. The nature of the substance in macrophages culture supernatant fluids was not determined. It appeared unlikely that it was antibody as it was not antigen specific and supernate-treated RBC were not agglutinated by anti-IgG or anti-kappa chain antisera.

Alter and Bach (1970) and Bach et al. (1970), in studies of the requirement for adherent cells in the production of a blastogenic response by antigen stimulated lymphocytes, also found that supernatant fluids of macrophage cultures would substitute for the cells themselves. Due to these observations Dutton and colleagues (1970) warned that synergistic effects may be due to an increase in the number of precursors stimulated or they could reflect an increase in the number of antibody forming cells derived from each precursor cell. Such might be the case if a nutritional requirement were supplied.

The ability of a 0.83 per cent ammonium chloride solution to lyse erythrocytes without damaging nucleated spleen cells was used by Leserman, Roseman, and Mosier (1971) to study the antigen dependent phase of the in vitro immune response. These authors reported that treatment of RBC stimulated spleen cell cultures with ammonium chloride one day after the beginning of culture abolished any PFC response observed after four additional days of incubation. Addition of more RBC

after the ammonium chloride treatment restored the PFC response. Ammonium chloride treatment on the third day of culture did not reduce the PFC response. Suspensions of adherent cells were incubated for 1 to 3 days with red cells and then treated with ammonium chloride. After washing of the macrophages non-adherent cells were added. No PFC resulted after five additional days of incubation unless the red cell antigen was replaced. These results demonstrated that although adherent cells were required for in vitro antibody production, they were unable to protect antigen from lysis by ammonium chloride.

Another approach to studying the requirement for macrophages in the in vitro immune response was taken by Sabet and Friedman (1970). They observed that addition of carbon particles to spleen cell cultures resulted in a suppression of the primary immune response to sheep erythrocytes. If carbon treatment was delayed for 48 hrs after the initiation of culture, less immunosuppression occurred. Treatment of donors of spleen cells with carbon in vivo 1 to 2 days prior to sacrifice also produced a subsequent suppression of the in vitro immune response.

Hoffman (1970) reported that the presence of peritoneal macrophages enhanced the PFC response of spleen cells studied by the Mishell and Dutton culture technique. He found that a critical ratio of PEC to spleen cells was required for maximum stimulation and that too many PEC would suppress the in vitro immune response. Peritoneal exuate cells could restore the ability of the non-adherent spleen cell population to respond to antigenic challenge.

While adherent cells appeared to be necessary for the in vitro response to erythrocyte antigens, Diener, Shortman and Russell (1970)

and Shortman and Palmer (1971) observed that macrophages were not necessary for an immune response to a polymerized form of Salmonella adelaide flagellin (POL). In fact, addition of peritoneal exudate cells to spleen cell cultures caused a decrease in the immune response to that antigen. Addition of anti-macrophage serum blocked the in vitro anti-sRBC response but did not affect the anti-POL response. Addition of PEC to macrophage-free spleen cells restored the responsiveness of the lymphocytes to sRBC. It appeared that macrophages released some nonsedimentable antigenic material from erythrocytes, due to the finding that culture supernates obtained from incubation of macrophages with erythrocytes could initiate immune responses in macrophage-free cultures of lymphocytes.

That the importance of the macrophage may vary with the antigen used has been pointed out by Unanue and Askonas (1967, 1968a, 1968b) and Unanue (1969). These workers have observed a necessity for macrophage participation in the immune response to Maia squinado hemocyanin (MSH), while macrophages were not necessary to elicit an immune reaction against the hemocyanin obtained from keyhole limpet (KLH). Aaskov and Halliday (1971) reported that in vitro investigations had revealed that macrophages were required for an immune response to pneumococcal polysaccharide. This was in disagreement with the results obtained indirectly through in vivo investigation (Howard and Siskind, 1969).

Conflicting reports on the role of the macrophage in the secondary immune response have come from in vitro studies. Pierce (1969) found that macrophages were not necessary to elicit an immune response in macrophage-poor cultures of spleen cells from mice primed with heterologous erythrocytes. Nevertheless, Pierce pointed out that the

cultures were not entirely free of macrophages and it could not be said with absolute certainty that the few remaining phagocytes in these cultures were not contributing to the immune response. Moore and Schoenberg (1968, 1971) reported that macrophages were not necessary for restimulation of primed lymphocytes and, in fact, actually attentuated the immune response when present. Conversely, Sabet and Friedman (1970) found that the in vitro secondary immune response to sRBC was suppressed by treatment of the cultures with carbon particles which presumably interfere with macrophage function. Others (Cosenza and Rowley, 1971; Cosenza, Leserman, and Rowley, 1971) observed a requirement for adherent cells in the secondary immune response.

Evidence that antigens retained by tissues are complexed to a small molecular weight RNA was first reported by Garvey and Campbell (1957) and Campbell and Garvey (1963). They found fragments of BSA complexed to RNA of liver macrophages several months after injection of the BSA antigen. These authors were the first to suggest that such antigen-RNA complexes might be important in responsiveness to antigens. This idea stemmed from their observation that antigen-RNA extracts were more sensitive in promoting anaphylaxis than was the original antigen alone.

Fishman (1959, 1961) and Fishman and Adler (1963) observed that lymph node cells would synthesize antibody against T_4 bacteriophage if they were exposed to macrophages or to RNA extracts of macrophages which had been preincubated with the phage particle. Further investigations by Fishman, Hammerstrom and Bond (1963) showed that the immunogenicity of the RNA extracts was specific for the type of phage used in the

macrophage sensitization step, and that the immunizing activity was sensitive to ribonuclease digestion.

Friedman, Stavitsky and Solomon (1965) discovered that RNA from macrophages sensitized with T_2 phage could elicit a secondary response when injected into mice primed with T_2. If the RNA preparation was treated with ribonuclease most, but not all, of the immunogenic properties were lost. This observation suggested that some of the antigenic components of the T_2 phage might still be present in the RNA preparation, and, indeed, Friedman and colleagues could detect such components by a sensitive complement fixation reaction. Hemocyanin labeled with radioactive iodine was found in immunogenic RNA fractions obtained from mouse peritoneal cells which had ingested labeled antigen by Askonas and Rhodes (1965).

Adler, Fishman, and Dray (1966), Fishman and Adler (1967) and Fishman, Adler and Holub (1968) described two types of immunogenic RNA which could be extracted from macrophages treated with antigens. One of the RNA fractions was free of any demonstrable antigen and was found to be responsible for mediating the 19S antibody response. The other RNA fraction contained both RNA and associated antigen. It was responsible for the production of the 7S antibody response. In addition, both the RNA and protein components of this second fraction were necessary for immunogenicity. Although it could be argued that the RNA eliciting the 19S response could contain antigen which was undetectable by present techniques, other observations made this possibility doubtful. First, these workers found that the antibody produced in response to this RNA fraction carried allotypic markers characteristic of the macrophage

donor, and second, if actinomycin was added to the macrophage cultures before antigenic stimulation, the resultant RNA extracts were capable of evoking only a 7S response. These two observations lent support to the hypothesis that RNA involved in the 19S response was newly formed by peritoneal exudate cells after antigenic stimulation and that it served as a messenger RNA. On the other hand, the RNA participating in the 7S response was thought to be preformed within the phagocytic cell.

Those who claim that macrophages produce a messenger RNA which programs lymphocytes to produce specific antibody have yet to prove that this RNA does not come from lymphocytes which are known to be present in peritoneal exudates. Schwartz, Ryder, and Gottlieb (1970) postulated an alternative to the messenger hypothesis to explain the observation that macrophages can direct allotypic specificity. They suggested that some as yet undescribed transfer RNA specific for the allotypic locus might be present in macrophages (or lymphocytes).

Pinchuck et al. (1968) discovered that C57BL/6 mice, which respond very poorly to a synthetic polypeptide, would produce antibody against that polypeptide if it was associated with normal macrophages or RNA from normal macrophages obtained from mice, rats or rabbits. Incubation of RNA obtained from macrophages treated with the polypeptide antigen with a second antigen did not render the second antigen immunogenic in mice injected with the RNA-antigen mixture.

Gottlieb, Glisin and Doty (1967) found that highly immunogenic ribonucleoprotein complexes were formed in macrophages which had ingested antigen. Antigen (T_2 phage) was present in an RNA fraction which had a unique banding density in cesium sulfate gradients, and T_2 phage

was associated with this RNA fraction only. The same RNA was present in both T_2-treated macrophages and macrophages incubated with a second virus. Both the immunogenic potential and the unique band seen in cesium sulfate gradients were lost after pronase digestion.

There have been some indications that new RNA synthesis results after macrophages ingest antigen. Halac, Rife and Renaldine (1964) observed a shift in the base composition of macrophage RNA after the cells were incubated in vitro with bovine serum albumin. Actinomycin D treatment of macrophages was shown by Bishop, Pesciotta and Abramoff (1967) to inhibit the production of a new 6-10S RNA found in antigen-treated macrophages. This RNA was shown to be responsible for enhancing the immunogenicity of antigen. Results of Cruchaud et al. (1970) were in direct disagreement with these reports. They reported that actinomycin D treatment of macrophages was only moderately inhibitory of the immunogenic potential of macrophage-associated antigen and, therefore, that RNA synthesis by macrophages must not be the only factor which was important in the response to macrophage-associated antigen.

The competitive hybridization experiments of Gottlieb and Straus (1969) provided evidence that the immunogenic RNA found in macrophages was not specific for antigen. RNA obtained from macrophages incubated with R17 phage could effectively compete for binding sites on DNA with RNA extracted from T_2 treated macrophages. Gottlieb (1969) determined from the small size (m.w. 22,000) of the RNA he studied, and from its relatively high guanine-cytosine content (58 per cent), that it was not a messenger RNA. Such a messenger would be too small to code for either the H or the L chain of immunoglobulins.

A caution to studies demonstrating binding of antigens to macrophage RNA has been put forth by Roelants and Goodman (1969) who determined that binding of antigens to RNA was an artifact brought about by linking anionic groups of antigens to RNA through Mg^{++} bridges. They observed that the formation of such complexes was unrelated to the potency of the antigen, did not depend on enzyme function, and did not require the synthesis of RNA after the introduction of antigen. In addition, there was a total absence of any relationship between immunogenicity and the capacity to form complexes with RNA.

Unanue and coworkers in a long series of articles have described experiments supporting the concept that macrophages function by carrying antigens on their cell membrane in high local concentration to lymphoid cells. These workers believe that antigen may not necessarily be actively "processed" by macrophages (Unanue and Askonas 1968a, 1968b; Unanue, Cerottini, and Bedford, 1969; Cruchaud and Unanue, 1971). Electronmicroscopic radioautographic studies of Unanue, Cerottini, and Bedford (1969) revealed localization of radiolabeled antigen in digestive vacuoles and on the surface of the macrophage. The material on the cell surface persisted up to 72 hrs without being incorporated into vacuoles. Membrane bound antigen was removed by treatment of the cells with trypsin or with EDTA. The antigenic material removed by these treatments was heterogeneous but of large molecular size. Because trypsin or EDTA treatment abrogated the immunogenic properties of the cells, these workers attributed the immunizing ability of antigen-macrophage complexes to the few molecules of antigen which were present on the surface of the cell (Unanue and Cerottini, 1970).

Schmidtke and Unanue (1971a) studied the uptake of radioactively labeled autologous and heterologous albumins by macrophages of normal mice. Both mouse and human albumin were engulfed and metabolized to the same extent by mouse peritoneal macrophages. The amount of antigen ingested was dependent on its physical form and not upon its foreignness to the macrophage donor. Most of the albumin was rapidly catabolized, but a small proportion remained associated with the cells both intracellularly and on the plasma membrane. They found that the antigen on the cell membrane was maintained independently of the intracellular albumin and that this membrane-bound material was responsible for the immunogenic potential of the macrophage-associated albumin.

When sheep red blood cells were used as antigen, Cruchaud and Unanue (1971) reported that antigenic material was released from macrophages into the extracellular fluid and that small amounts of ^{125}I-labeled antigen could be found on the cell surface. The cell-associated antigen was more immunogenic than that released into the medium. Release of non-protein bound ^{125}I indicated that the red cells were catabolized intracellularly, but it was also apparent that some molecules of the antigen escaped extensive catabolism and became associated with the cell membrane where they were available for recognition by lymphoid cells.

The controversey over the role of the macrophage in the induction of immunity is still unsettled. It is not clear whether macrophages provide a specific messenger RNA for antibody synthesis; a highly unlikely possibility due to the fact that macrophages themselves do not produce antibody (Adler, Fishman and Dray, 1966). On the other hand, antigen may combine specifically with an RNA which is induced by contact

with the antigen or nonspecifically with an RNA which is preformed in the macrophage. Such antigen-RNA complexes may then possess enhanced immunogenic properties. It was suggested by Braun (1969) that RNA alone may act as an activator or a repressor of lymphocytes, but that when antigen is complexed with the RNA, the antigen acts to guide the RNA-activator to the proper cell. In this regard, it is interesting to note that Campbell and Kind (1971) have recently reported that polynucleotide adjuvants act by stimulating the precursor of the antibody forming cell.

Lymphoid Cells

For some time immunologists have recognized that different types of immunity appear after exposure of an animal to foreign antigen. Different types of hypersensitivity were initially described by differences in the time of appearance of skin test reactions upon challenge with antigen. Prausnitz and Kustner (1921) were successful in transferring immediate hypersensitivity to fish with the serum of a sensitive donor. Helmholtz (1909) passively transferred delayed hypersensitivity to tubercle bacilli with defibrinated blood of infected guinea pigs. Later, Chase (1945) determined that the lymphocyte was responsible for passive transfer of delayed-type hypersensitivities. Passive transfer experiments thus provided a firm foundation for the belief that there was some sort of compartmentalization of the immune response.

The separation of delayed-type from immediate-type immune reactions became more apparent upon the discovery by Coons, Leduc and Connally (1955) that the plasma cell was responsible for antibody formation, while the small lymphocyte was implicated in reactions of homograft immunity (Gowans, McGregor and Cohen, 1963) and the graft-versus-host

reaction (Heldemann, Linscott, and Marlino, 1962).

One of the most important discoveries in the field of cellular immunology was made by Miller (1961) who found that neonatal thymectomy in mice prevented development of cell-mediated immunity and some humoral antibody responses. Previously it was believed that removal of the thymus in adult life was inconsequential. However, Miller (1965) observed a slow waning of immunological competence in adult animals after thymectomy. Immune impairment similar to that seen in neonatally thymectomized animals resulted from removal of the thymus followed by letahal irradiation and bone marrow reconstitution (Miller, Doak, and Cross, 1963). Because the thymus contained few, if any immunocompetent cells, as judged by its lowered ability to induce a graft-versus-host reaction or to adoptively transfer antibody formation, the concept of the "central" lymphoid tissue arose (Playfair, 1971b). Central lymphoid tissue was defined as any organ which plays a critical role in the early differentiation of cells toward immunological competency but which is not itself engaged in immune responses. The central lymphoid tissue supply competent cells to the "peripheral" lymphoid tissue where these cells further differentiate and divide upon antigenic stimulation and where immune reactions manifest themselves.

Glick, Chang and Japp (1956) were first to report that the avian bursa of Fabricius played an essential role in the development of humoral immunity in that species. Further studies with chickens (Warner and Szenberg, 1962, 1964; Jankovic and Isvaneski, 1963) provided evidence that the avian thymus exerted its influence on the development of small lymphocytes engaged in cellular immunity. Thus, the chicken was found

to have two central lymphoid organs, the thymus and bursa of Fabricius.

In mammals, the organ which functions as does the bursa in fowl has not been identified, but it is speculated to be some gut-associated, lympho-epithelial tissue (Archer, Sutherland and Good, 1963). Experimental evidence that the gut-associated lymphoid tissue of rabbits may be equivalent to the bursa of the chicken was provided by the work of Cooper and coworkers (1968).

Lymphoid cells which are influenced in their development by the thymus have been given the names, thymus-dependent cells, or T-cells. Those cells which require the bursa of Fabricius for development (as in the chicken) or which originate from the bone marrow of mammals have been named bursa-dependent cells, bone marrow-derived cells, or B-cells.

Evidence for Cellular Cooperation in the Immune Response

Evidence for a cooperative effect between thymus-derived lymphocytes and bone marrow-derived lymphocytes in the production of a humoral immune response to certain antigens, such as heterologous erythrocytes and serum proteins has accumulated recently (Claman and Chaperon, 1969; Miller and Mitchell, 1969; Miller, Basten, Sprent, and Cheers, 1971; and Playfair, 1971b). These investigations have included studies of hapten-carrier effects, consequences of neonatal thymectomy, and participation of T-cells and B-cells during *in vivo* and *in vitro* immune responses.

The carrier effect. By definition a hapten is not immunogenic by itself but combines with antibody produced against it when it is coupled to a macromolecular carrier. Brownstone, Mitchison and Pitt-Rivers (1966) reported that optimal secondary responses to haptens

only occurred in animals immunized with the carrier used in primary immunization. The protein carrier did not contribute to the binding energy of the hapten with antibody suggesting that anti-hapten antibody did not distinguish structural characteristics of the carrier molecule. This fact was difficult to explain with current theories of antigen recognition unless one assumed that two or more cells were involved; one cell recognizing the haptenic determinant and others recognizing determinants on the carrier molecule.

Other investigators reported that preimmunization of an animal with a hapten-carrier complex would increase the antibody response to a second carrier if that carrier was complexed to the original hapten. In addition, if two haptenic determinants were used in such experiments, a better response occurred (Dixon and Maurer, 1955; Weigle 1962, 1964, 1965a, 1965b; Linscott and Weigle, 1965; Dietrich, 1966; Plescia et al., 1968; Leskowitz, 1968; Rajewski, 1969). The enhancement seen with multiple determinants brought about the proposal that there might be a requirement for cellular interactions in the immune response. If this were the case each additional determinant would serve to increase the possibility of such cellular encounters. It was apparent that cells specific for one determinant could interact with cells recognizing another determinant and that such interactions would increase the responsiveness of the second cell.

In 1967, Benacerraf, Green and Paul noted a genetic defect in strain 13 guinea pigs which resulted in their inability to mount an antibody or delayed hypersensitivity response to a DNP-poly-lysine complex. Normal responses to DNP occurred in these animals if the DNP

hapten was coupled to a BSA carrier. A similar observation in rabbits was reported by Rajewsky et al. (1967) who observed that some rabbits could not make antibody against one of the two subunits of the lactic dehydrogenase enzyme. If the hybrid molecule was injected into these animals, antibody was produced against both subunits. Animals immunologically tolerant of the carrier subunit did not produce detectable antibody after immunization with the hybrid enzyme. Thus, antibody production in the studies of Benacerraf et al. and Rajewsky et al. required the simultaneous recognition of both the hapten and the carrier molecules.

It was determined by Mitchison (1967) that the carrier and the hapten did not act as parts of a single determinant. He placed "spacer" molecules between the hapten and the carrier and found that this insertion did not affect the immunogenicity of the complex.

The clearest evidence that the hapten-carrier effect is due to the interaction of two different cells came from cell transfer studies. Spleen cells from mice primed to a hapten-carrier complex were not effective in producing an immune response after transfer to irradiated recipients if they were challenged with the hapten on a different carrier. Responsiveness was restored if the animals were given spleen cells from another donor which was primed with the carrier used in the challenge.

Transfer of lymphoid cells from bovine gamma globulin (BGG) primed guinea pigs to DNP-ovalbumin-primed guinea pigs was reported by Paul and coworkers (1970) to markedly enhance the secondary anti-DNP response of these animals when challenged with DNP-BGG. If the BGG primed cells were obtained from animals rendered immunologically unresponsive to BGG no enhancement of the anti-DNP response occurred.

Hapten-carrier effects have been observed in in vitro experiments as well as in the in vivo studies reported above (Katz, Bluestein, Rouques, and Pierce, 1971; Segal, Globerson, and Feldman, 1971a, 1971b; Kunin, Shearer, Segal, Globerson and Feldman, 1971; Kettman and Dutton, 1971; Falkoff and Kettman, 1972). These reports have also pointed to the need for cellular cooperation in the immune response.

Segal et al. (1971a, 1971b) and Kunin et al. (1971) determined that in vitro production of a primary response to alpha-DNP-poly-L-lysine was inhibited by free DNP-lysine or free poly-L-lysine, demonstrating the presence of specific cellular receptors for both the hapten and the carrier. Preimmunization of animals in vivo with carrier enhanced the primary response against the hapten by spleen cells in vitro. This effect was carrier specific. If vinblastine was given to the animal simultaneously with the carrier preimmunization, the subsequent in vitro response to the DNP-carrier complex was abolished. However, administration of vinblastine 24 hrs after carrier pretreatment had no effect. These authors proposed that the carrier effect may occur due to the stimulation of just one cycle of DNA replication. They suggested that this replication cycle might be necessary for the production of more carrier specific receptors.

It was reported by Segal et al. (1971b) that spleen cells from rabbit serum albumin (RSA) tolerant animals did not respond to RSA-DNP but did respond to hemocyanin-DNP. Pretreatment of RSA tolerant spleen cells with DNP-RSA three hours prior to application of alpha-DNP-poly-L-lysine abrogated a subsequent anti-DNP response. This provided evidence for the presence of functional receptors for DNP on the antibody forming

cell in the carrier tolerant animal. Tolerance could be attributed to a separate cell which only responds upon contact with the carrier molecule.

Kunin and coworkers (1971) studied the role of thymus-derived and bone marrow-derived cells in the hapten-carrier phenomenon. Antibody to DNP was not produced in vitro by spleen cells of lethally irradiated animals which had been reconstituted with bone marrow cells or thymus cells alone and then primed with carrier. If the irradiated animals were treated with RSA and T-cells 6-8 days before injection of bone marrow cells, the carrier effect occurred in cultured spleen cells. This effect was not observed when RSA and bone marrow were administered 6-8 days before an injection of thymus cells, however. These results suggested that an initial interaction of carrier with thymus-derived cells was necessary before the bone marrow cell could be stimulated to produce antibody.

Similar carrier effects were observed by Kettman and Dutton (1971) when spleen cells were immunized in vitro with TNP coupled to red blood cell carriers. They noted that spleen cells from carrier-primed animals would enhance the anti-hapten response of normal spleen cells when the two cell types were mixed in culture. These workers determined that the carrier primed cells were thymus-derived, and that once these cells were primed, they were resistant to irradiation (Kettman and Dutton, 1971; Falkoff and Kettman, 1972).

The premium effect. Celda (1967), in studies of the secondary immune response to human serum albumin (HSA), discovered that the anti-HSA response increased disproportionately with the number of spleen cells

transferred to irradiated recipients. He called this phenomenon "the premium effect". Later, Gregory and Lajtha (1968) reported that although the number of hemolytic foci to sRBC increased linearly with the number of spleen cells transferred to irradiated animals, the PFC response increased out of proportion to the size of the spleen graft. Since it has been clearly shown that the number of PFC detected in a suspension of immunized cells is linearly related to the number of cells plated (Jerne, Nordin and Henry, 1963) and that this number is not affected by the presence of unimmunized cells (Celda, 1967), synthesis and release of antibody is considered to be an independent function of a single cell. Gregory and Lajtha emphasized that since the rate and number of divisions of PFC precursors increased with increasing chance for cellular interactions, the production of PFC from their precursors could be attributed to a cooperative process. A similar effect was reported by Bussard and Lurie (1967) who observed that the total number of PFC produced in vitro by peritoneal cells increased disproportionately to the number of cells in culture.

Immune response in thymus-deprived animals. The thymus gland is known to be essential for the normal development and function of lymphoid tissue, and especially for the ontogeny of immune responses which are considered to be cell mediated (Miller, 1961, 1964; Miller and Osoba, 1967). In addition to the depressive effect that neonatal thymectomy has on cellular immunity, this treatment has resulted in depression of humoral immune responses against sheep erythrocytes (Basch, 1966; Friedman, 1965; Humphrey, Parrott and East, 1964; Miller, deBrough, and Grant, 1965), ovalbumin (Arnason, DeVaux St. Cyr, and Schaffner, 1964)

and BSA (Arnason et al., 1964; Taylor, 1963). Lowered responses due to lack of T-cells have been borne out in studies of the immune response of congenitally thymusless "nude" mice (Kindred, 1971; Reed and Jutila, 1972). Responses of thymectomized mice to other antigens such as tetanus toxoid (Hess, Cottier, and Stonar, 1963; Hess and Stonar, 1966), pneumococcal type III capsular polysaccharide (Fahey, Barth and Law, 1965; Humphrey et al., 1964), Salmonella flagellar antigen (Pinnas and Fitch, 1966), ferritin (Fahey et al., 1965) and hemocyanin (Fahey et al., 1965; Humphrey et al., 1964; and Unanue, 1970) are not different from that of sham operated controls.

Neonatal thymectomy may severely depress but not completely eliminate the humoral antibody response under study. Masser, Cooper, and Good (1967) and Masser, Good and Cooper (1970) reported that a combined treatment of neonatal thymectomy followed by irradiation (Tx-X) produced an even greater depression of the anti-erythrocyte response when animals were challenged in adult life as compared to animals who were only thymectomized as neonates. On the other hand, Tx-X animals responded as did normal or sham thymectomized animals upon challenge with Brucella abortus or Salmonella typhosa.

The effect of neonatal thymectomy on the immune response has been overcome by increasing antigen doses one hundred fold (Sinclair, 1967; Sinclair and Elliott, 1968; Lemmel, Cooper and Good, 1971; Aird, 1971). These data implied that thymus-derived cells were not obligatory for the immune responses which were depressed in thymus-deprived animals, but that these cells merely served to amplify the response. On the other hand, Playfair (1971b) suggested that the delayed immune responses

resulting from increased antigen doses may occur due to an expansion of the small number of thymus cells which had peripheralized prior to the time of thymectomy.

In vivo cellular cooperation. The first direct evidence for the participation of thymus-derived lymphocytes and bone marrow-derived lymphocytes in the immune response came from the work of Claman and co-workers (Claman, Chaperon, and Triplett, 1966a, 1966b; Claman and Chaperon, 1969). These investigators reported that reconstitution of lethally irradiated recipients with thymus cells and bone marrow cells followed by injection of an immunizing dose of sRBC resulted in immune responses which were higher than the additive response of either cell type was given alone. They called this phenomenon bone marrow cell-thymus cell synergism.

Claman, Chaperon, and Selner (1968) observed that the thymus-dependent cell must be living. Transfer of sonicated or irradiated thymus cells along with normal bone marrow cells was ineffective in synergy experiments.

The experiments of Miller and Mitchell (1967), Mitchell and Miller (1968a, 1968b), and Miller and Mitchell (1969) confirmed the observation of Claman and colleagues that two types of lymphoid cells were required for the anti-sheep erythrocyte response. They reported that a significant plaque forming cell response occurred in the spleens of lethally irradiated mice which had been given bone marrow cells, sRBC and spleen cells from donors which were irradiated and injected with thymus cells and sheep erythrocytes one week previously. No responses occurred in recipients if the primary recipient was injected with thymus

cells only. Responses were also negative in secondary recipients when bone marrow cells were not inoculated.

Miller and Mitchell (1968) reported that the anti-sRBC response of neonatally thymectomized animals was restored to normal levels by injections of normal syngeneic or allogeneic thymus or thoracic duct lymphocytes. There was no significant increase in the anti-sRBC response of thymectomized mice if they were given bone marrow cells, irradiated thymus cells, irradiated thoracic duct cells, thymus extracts, or yeast cells. When the recipient was reconstituted with semi-allogeneic cells, anti-H-2 antiserum treatment of the recipient spleen cells just prior to determination of the PFC response allowed identification of the antibody forming cells as being of host origin. Cross, Leucars, and Miller (1964) reported that normal spleen cells, but not spleen cells of thymectomized donors, would restore the immune response in mice which had been thymectomized, irradiated and bone marrow protected.

Nossal, Cunningham, Mitchell and Miller (1968),using the fully syngeneic CBA and CBA/T6T6 donor-recipient combination, observed that neonatally thymectomized mice were rendered responsive to sRBC immunization by transferred thymocytes. The antibody forming cell was of the recipient type as judged by chromosome analysis. Likewise, in lethally irradiated animals reconstituted with mixtures of chromosomally marked bone marrow or thymus cells, the antibody forming cells were determined to originate from the bone marrow inoculum.

Miller and coworkers concluded from their experiments that in the mouse the precursor of the antibody forming cell (AFC) was of bone marrow origin, but that a specific thymus cell-antigen interaction must

occur which in some manner potentiates the differentiation of the AFC precursor. This theory was substantiated by the results of Shearer and Cudkowicz (1969) who found thymic antigen reactive cells (ARC) which were capable of initiating the anti-sRBC response in this species. They noted, in addition, that the ARC must interact specifically with antigen.

Normal bone marrow cells adoptively transferred immunity to sRBC to irradiated rabbits in the experiments of Abdou and Richter (1969a) and Richter, Rose and Abdou (1970). The bone marrow cells of the rabbit were identified as the ARC when Richter and Abdou (1969) discovered that in the x-irradiated rabbit, host cells were the precursor of the AFC. Abdou, Rose and Richter (1969) reported that the bone marrow ARC was radiosensitive. These workers (Abdou and Richter 1969b, 1970b) observed that if bone marrow cells were harvested 24-48 hrs following immunization they were unable to transfer immunity to the antigen used for priming. These same cells were fully capable of adoptively transferring immune responses to other antigens. They postulated that ARC migrated out of the bone marrow after antigenic stimulation and transferred information to the AFC in some other organ. Abdou and Richter (1970c) identified cells in the rabbit sacculus rotundus as the AFC in that species.

The phenomenon of thymus cell-bone marrow cell collaboration in avian species has not been extensively investigated, however a few recent reports have indicated that such cellular collaboration may occur. Gilmour, Thies and Thorbecke (1970) transferred cells (bursal, thymus or spleen) from chickens isogeneic for the strong B histocompatibility locus to irradiated chicks one day after hatching. They found that only spleen cells were effective in transferring the sRBC response when

antigen was given immediately after transfer. In tests of synergism by mixtures of cell populations, they observed a modest augmentation of the sRBC response when animals were reconstituted with bursal cells and spleen cells as compared to spleen cells alone. McArthur, Gilmour, Hochwald, and Thorbecke (1971) pretreated spleen cells with anti-thymus serum before transfer and were no longer able to adoptively transfer the primary sRBC response to x-irradiated, recipient chickens. Synergistic effects were noted by mixtures of chicken bone marrow and thymus cells if cell doses were lower than the threshold dose for consistent PFC responses when the cells were administered singly (McArthur, Gilmour and Thorbecke, 1972). The evidence for thymus-bursal cell synergy was only suggestive. These investigators proposed that the reason for this observation might be the variable background responses of bursal cells along with the lack of a third cell type required in this system.

Rouse and Warner (1972) reported reduced antibody titers to horse erythrocytes and DNP-BSA in chickens deprived of thymus-dependent cells by a treatment which combined neonatal thymectomy with anti-chicken thymus serum. Thymus-deprived chickens responded to injections of Brucella with agglutinin titers which were not significantly different from controls.

In vitro cellular cooperation. The in vitro antibody production technique of Mishell and Dutton (1967) has been utilized extensively in studies of thymus cell-bone marrow cell collaboration during the immune response. Such investigations have further substantiated the phenomenon of thymus-bone marrow cooperation originally observed in studies of employing reconstituted, irradiated animals.

Spleen cells from thymus-deprived mice do not respond *in vitro* to sRBC (Mosier, Fitch, Rowley and Davies, 1970; Munro and Hunter, 1970; Rosenoer, Biano and Brown, 1970; Hirst and Dutton, 1970; Aden, Reed, and Jutila, 1972). Mosier *et al.* (1970), Rosenoer *et al.* (1970) and Hirst and Dutton (1970) determined that the cellular defect in thymectomized mice was in the non-adherent cell population. Others (Doria, Martinoz, Agarossi and Dipietro, 1970; Munro and Hunter, 1970; Aden, Reed and Jutila, 1972) reported that addition of thymocytes to spleen cell cultures of thymus-deprived mice restored responsiveness toward sheep erythrocytes.

Hartmann (1970, 1971) prepared thymus-dependent cells from spleens of irradiated mice one week after injection of thymus cells and sheep erythrocytes (educated T cells). Bone marrow-derived cells were obtained from spleens of thymectomized, irradiated, bone marrow-grafted animals. Both cell types were necessary for an *in vitro* response to sRBC. He found that the T-cell must be "educated" with the specific antigen used for *in vitro* stimulation. When T-cells and B-cells were cultured with two different RBC antigens, one of which was used to educate the T-cell, a PFC response occurred against both erythrocytes. This observation was confirmed by Hunter, Munro and McConnell (1972) and by Kettman and Dutton (1971). The number of PFC detected after 3 to 4 days of culture was directly proportional to the number of T-cells added to the culture. Thymus-dependent lymphocytes were found to promote clone expansion, since higher concentrations of T-cells led to shorter doubling times for PFC.

Vann and Kettman (1972) confirmed the observation of Hartmann

(1970, 1971) and further reported that bone marrow-derived cells could be inactivated by exposure to 2000 R gamma-irradiation while educated T-cells were not. This result was in agreement with those of Katz et al. (1970) and Kettman and Dutton (1971) who reported radioresistance of carrier-primed cells. Using TPN-sRBC, Vann and Kettman found that responses occurred against the DNP determinant to which neither the T-cell nor the B-cell populations had been previously exposed. In these experiments sheep erythrocyte "educated", thymus-dependent cells were analogous to carrier-primed spleen cell populations.

Consequences of anti-theta antiserum treatment. Antiserum prepared against the theta antigen found on thymocytes of mice has been used to study the various functions of thymus-derived lymphocytes. Generally, such experiments involved treating cells in vitro with anti-theta serum and complement followed by assaying the immune competence of the treated cell suspensions in vitro or in adoptive hosts. Several investigators have reported that immune plaque forming cells or their precursors were not directly affected by anti-theta serum (Greaves and Moller, 1970; Takahashi, Carswell and Thorbecke, 1970; Cerottini, Nordin and Brunner, 1970; Raff, 1971; Chan, Mishell and Mitchell, 1970). Schimpl and Wecker (1970) observed a reduction of the in vitro response of spleen cells to sRBC when the cells were treated with anti-theta and complement prior to culture. This response was partially restored by the addition of thymus cells to the anti-theta treated spleen cell cultures. Chan et al. (1970) reported similar observations and determined that the response of spleen cells treated with anti-theta could be restored by the addition of "educated" thymus-derived cells.

The studies of Takhashi, Carswell and Thorbecke (1970, 1971) revealed that anti-theta antiserum treatment abolished the ability of mouse spleen cells to transfer both primary and secondary responses against sRBC to irradiated recipients. They obtained partial reconstitution of the primary response by addition of normal thymocytes to anti-theta treated spleen cells. Normal thymus cells did not reconstitute the secondary response, however. Primary and secondary responses against Brucella abortus were not reduced by treating spleen cells with anti-theta serum.

Raff (1970) reported that theta-positive cells were required for secondary responses to hapten-carrier protein conjugates. Treatment of primed helper cells with anti-theta markedly depressed the anti-NIP response when these cells were transferred to irradiated animals challenged with BSA-NIP. Treatment of NIP-chicken gamma globulin primed cells had no effect on the secondary adoptive anti-NIP response. These results demonstrated that the role of theta-positive cells in the immune response was one of specific antigen handling rather than secretion of antibody.

The Mechanism of Cellular Collaboration

Several theories have been proposed to explain the mechanism whereby thymus-dependent cells assist bone marrow-derived cells during initiation of antibody synthesis. Talmage, Radovich and Hemmingsen (1969) suggested two different three cell models for antibody formation. In both models the adherent cell was capable of binding antigen. This cell's function was to bring the two non-adherent cells together. One model proposed that the thymus-dependent cell was antigen specific and

made a messenger RNA which was transferred to the bone marrow-derived cell. The B-cell then provided the ribosomes and synthesized antibody. Abdou and Richter (1970a) also speculated that antigen specific ARC transferred specific information to uncommitted antibody forming cell precursors. The second model of Talmage et al. stated that the T-cell was a delayed hypersensitivity cell which upon contact with specific antigen greatly enhanced the interaction of macrophage and bone marrow-derived cell.

There is no experimental evidence to support the contention that ARC or T-cells transfer specific information to antibody forming cells via RNA. It is known that both the subclass of immunoglobulin and allotype of antibody formed are characteristic of the B-cell and that thymus cells are not specialized for molecular class of antibody (Klein and Herzenberg, 1967; Shearer, Cudkowicz and Priore, 1969a, 1969b, 1969c; Shearer and Cudkowicz, 1969; Cudkowicz, Shearer and Priore, 1969). Although this does not rule out the possibility that the T-cell may transfer a limited amount of information to the B-cell, it makes it highly unlikely that the T-cell alone determines the structure of the immunoglobulin produced. The T-cell could, however, transfer the information for the variable region of H and/or L chains where antibody specificity is determined.

It was proposed by Mitchison (1969b, 1969c) that thymus-dependent lymphocytes produced a nonsecretable immunoglobulin which he named IgX. He suggested that antigen was bound to the IgX receptors on the thymocyte surface. This interaction would allow the antigen to be presented to B-cells in the proper concentration to trigger antibody

formation. Mitchison's model required that determinants recognized by B-cells and T-cells must be associated with the same molecular structure.

Miller et al. (1971) determined that triggering of unprimed B-cells could not be accomplished by antigen coated tolerant T-lymphocytes. Treatment of primed or unprimed T-cells with mitomycin C impaired their "helper" activity, suggesting that T-cells must be activated by antigen to differentiate and during this process they may produce some factor essential for triggering B-lymphocytes.

Surface membrane interactions between specialized receptors on B-cells and T-cells, other than antigen-specific receptors, have been speculated by Dutton et al. (1971) to be another alternative to explain thymus cell-bone marrow cell collaboration. Such interactions would be facilitated by antigen bridges between immunoglobulin receptors on B and T lymphocytes.

Bretscher and Cohn (1968, 1970) proposed that thymus-dependent lymphocytes secrete an as yet unidentified antibody called "carrier" antibody. They suggested that induction of humoral antibody formation involved the recognition of two determinants of an antigen; one by receptor antibody molecules on the surface of the AFC and a second by carrier antibody. If antigen alone was recognized by the AFC receptor, paralysis would ensue. Recent reports in the literature have lent support to this hypothesis (Kennedy, Treadwell, and Lennox, 1970; Feldman and Basten 1972a, 1972b).

Kennedy, Treadwell and Lennox (1970) observed synergism in anti-erythrocyte responses between bone marrow and peritoneal cavity cells when both cell types were used to reconstitute irradiated

recipients. They noted that heat-killed peritoneal cells or extracts of these cells were also effective. The phenomenon was determined to be antigen specific. Because peritoneal cavity cells or their extracts were not immunogenic when tested in primed animals, they believed that antigen was not present in their preparations and that the effect noted was due to some sort of immunoglobulin.

In 1971, Feldmann and Basten reported that thymocytes were not required for in vitro immune responses to polymerized flagellin but that T-cells were necessary to elicit an immune response to monomeric flagellin or erythrocytes coated with monomeric flagellin. The fact that comparable levels of antibody forming cells to these three forms of the same antigen occurred in normal or x-irradiated, bone marrow grafted animals, suggested that the mechanism of immune induction was the same for each antigen form. Further studies using a hapten determinant coupled to a thymus-independent carrier (polymerized flagellin, POL) or a thymus-dependent carrier (donkey red blood cells, dRBC) showed that immune responses in adult thymectomized, bone marrow repopulated mice only occurred against DNP-POL. It was apparent, therefore, that thymus-dependency was related to the mode of presentation of antigenic determinants rather than to their specificity. These observations seemed to fit the hypothesis presented by Mitchison (1968, 1969) that T-cells stimulate B-cells through an antigen "focusing" mechanism. However, further investigations by Feldmann and Basten (1972a, 1972b) revealed that when carrier and hapten primed T-cells and B-cells were placed in separate compartments on either side of a cell impermeable membrane along with antigen, excellent cooperative responses occurred. They

noted that the restorative effect of the thymus-derived cell product was antigen specific. Feldmann and Basten (1972b) reported that T-cell helper activity required no further cell division after initial priming, but that it was abolished by inhibitors of RNA and protein synthesis. Feldmann (1972b) has determined that thymus-bone marrow interactions do not occur in the absence of macrophages. Feldmann and Basten suggested that thymus-dependent cells elaborate an IgX which is complexed with antigen. They believe that IgX-antigen complexes may bind to the surface of macrophages where a lattice of repeating antigenic determinants is formed. Bone marrow-derived PFC precursors would then be stimulated when they encountered antigen having repeating determinants. Schmidtke and Unanue (1971b) found that B-cells preferentially bind to macrophages, and the ultrastructural studies of Miller and Avrameas (1971) pointed to a close association between macrophages and cells synthesizing antibody.

Another model for thymus cell-bone marrow cell cooperation which is supported by some experimental evidence postulates the existence of a chemical mediator elaborated from thymus dependent cells (Dutton et al., 1971). This scheme proposes that a B-cell is stimulated after it receives two signals. The first signal occurs upon the interaction of antigen with receptor molecules on the surface of the AFC precursor. The second signal is a diffusable chemical mediator which results from the interaction of antigen with thymocyte receptors. The chemical mediator is itself nonspecific, its only limitation being the effective distance of its diffusion gradient. The experiments of Hartmann (1970) and Kettman and Dutton (1971) showed that when

thymus-dependent cells "educated" with antigen x which were cultured along with bone marrow-derived cells they responded to antigen y provided that both antigen x and antigen y were present.

A second type of experiment has been reported in which chemical mediators may be liberated in larger quantity. Such a case arises when allogeneic, non-adherent cells are used to restore immune responses of bone marrow-derived cells. Hirst and Dutton (1970) found that allogeneic spleen cells were more effective than syngeneic cells in restoring the in vitro immune response of spleen cells taken from neonatally thymectomized mice. The effect of allogeneic cells was even greater if these cells were x-irradiated prior to addition to the spleen cell cultures. Dutton and coworkers (1971) suggested that in this system a chemical mediator was produced by T-cells in response to the histocompatibility antigens present on the B-cells. They proposed that irradiation inhibited the cytotoxic activity of T-cells but did not inhibit the production of this nonspecific chemical mediator. The work of Hirst and Dutton was confirmed by the studies of Kennedy and Ekpaha-Mensah (1971). Analogous results have arisen from the observation of Katz, Paul, Goidl and Benacerraf (1971) that a graft-versus-host (GVH) reaction would stimulate an anti-DNP response in ovalbumin-DNP primed mice in the absence of further antigenic challenge. McCullagh (1970b) observed that transfer of allogeneic lymphocytes abrogated tolerance of rats unresponsive to sRBC. Because the PFC detected were mainly of host origin, it appeared that the GVH reaction produced by the allogeneic cells had liberated chemical mediators capable of stimulating nonresponsive host lymphocytes.

Cellular Sites of Immunologic Unresponsiveness

Delineation of the cells required for induction of immunity provided a foundation for further investigations of the cellular sites of immunological tolerance. These studies have located the cellular "lesions" which may occur in the unresponsive animals. Hopefully, the subcellular mechanism of immune tolerance will soon be elucidated.

The Macrophage

Early attempts at tolerance induction were only successful in neonatal animals (Smith, 1961). This fact, along with the observation that neonates had an immature reticuloendothelial system (Karthigasu et al., 1965; Reade and Jenkin, 1965) led to speculation that inability of macrophages to process antigen may be the critical event in tolerance induction.

Mitchell and Nossal (1966) reported that cells of newborn animals did not phagocytose ^{125}I-labeled salmonella flagellin and that this antigen was distributed diffusely throughout the lymphoid tissue of the neonate, including the thymus. This finding was in contrast to results in adult animals where antigen was phagocytized efficiently and was concentrated in lymphoid follicles. Others (Argyris, 1968; Martin, 1966) reported that poor immune responses in neonates and ease of tolerance induction could be attributed to lack of phagocytic function.

Ada, Nossal and Pye (1965) studied the localization of S. adelaide flagellin in lymphoid organs of normal and tolerant animals. They found that tolerant animals handled antigen in a manner comparable to normal controls. Similar observations were reported by Humphrey and Frank (1967).

Peritoneal cells from normal rabbits or rabbits tolerant to BGG were reported by Harris (1967) to be equally able to engulf antigen and stimulate DNA synthesis of spleen cell suspensions of immune rabbits. Mitchison (1969a) determined that peritoneal exudate cells of BSA paralysed donors were as effective as PEC of normal donors in processing BSA antigen for subsequent immune responses upon transfer to normal recipients.

Although the macrophage was shown to be fully functional in tolerant animals, it has been determined that procedures which cause decreased phagocytosis favor tolerance induction. The physical state of antigen has been found to influence the ability of that material to induce immunity or tolerance. Soluble antigens are generally more tolerogenic and less well phagocytized than are particulate antigens. Aggregate-free preparations of protein solutions prepared by high speed ultracentrifugation (Dresser, 1962; Gamble, 1966; Weigle and Golub, 1967; Biro and Garcia; 1965) or by "biological filtration" (Frei et al., 1965; Golub and Weigle, 1969) are highly tolerogenic. Conversely, these same antigens when in an aggregated state favoring their uptake by phagocytes are highly immunogenic (Biro and Garcia, 1965). Neill (1970) reported that treatment of BALB/c mice with RES depressants enhanced induction of tolerance or a hyporesponsive state to human gamma globulin. This observation supported the contention that if antigen is not processed appropriately by macrophages tolerance may ensue.

The Lymphocyte

Early reports of a specific inability of lymphocytes derived from immunologically tolerant donors to transfer adoptive immune

responses to x-irradiated recipients pointed out the lymphocyte as the cellular site of immunologic unresponsiveness (Dietrich and Weigle, 1964; Denman, Vischer and Stastney, 1967). Since the discovery that T-cells and B-cells may participate in humoral immune responses, much effort has gone into studies designed to test which of these cells becomes tolerant in the unresponsive animal.

Tolerance in thymus cells has been observed by several investigators. Isakovic et al. (1965) were able to restore immune responses of thymectomized, irradiated, bone marrow protected mice with normal thymus grafts. Thymus grafts from tolerant animals did not enable the recipient to respond to the tolerated antigen. Taylor (1968, 1969) found that thymus cells obtained from donors treated 24 hrs previously with 10 mg BSA would not cooperate with normal bone marrow cells in the anti-BSA response. Bone marrow cells taken from the same tolerogenized donors were fully competent to elicit an antibody response to BSA when in combination with normal thymus cells. Tolerance in thymocytes taken from mice treated with sRBC plus cyclophosphamide was reported by Many and Schwartz (1970), while no tolerance was observed in the bone marrow cell population of these animals. Miller and Mitchell (1970) obtained cyclophosphamide-induced tolerance in peripheralized, thoracic duct, thymus-dependent lymphocytes, but they could not detect unresponsiveness in lymphocytes resident within the thymus. They reported further that bone marrow cells of their sRBC tolerant animals were fully capable of cooperating with normal thymus-derived cells in the anti-sheep erythrocyte response. Inability to render bone marrow cells unresponsive by treatments which employ cyclophosphamide may be explained by the

fact that antigen initially stimulates T-cells (Davies et al., 1966). Cyclophosphamide affects cells which have been induced to proliferate. Because the T-cell helper function is required for B-cell stimulation, initial suicide of T-cells leaves B-cells unstimulated and unaffected by the drug.

Playfair (1969) induced tolerance in (NZB x BALB/c)F_1 mice using an erythrocyte-cyclophosphamide tolerance inducing regimen. He could not detect suppression of thymocytes in these experiments. In a later communication, Playfair (1971a) reported that tolerance could be induced in thymus cells of BALB/c or young (NZB x BALB/c)F_1 hybrids but not in older F_1 hybrids, NZB adults, or young NZB mice.

Many of the inconsistencies observed in tolerance induction of bone marrow cells have been explained by the work of Chiller and colleagues (Chiller and Weigle, 1970; Chiller, Habicht, and Weigle, 1970, 1971; Weigle, Chiller and Habicht, 1972). In initial experiments, Chiller and coworkers (1970) assayed bone marrow and thymus cells taken from mice injected 17 days previously with deaggregated human gamma globulin (soluble HGG, sHGG) for their ability to cooperate with their normal counterparts in the anti-HGG response. They found that thymus cells and bone marrow cells of sHGG-treated donors were unable to synergize with normal cells in the anti-HGG response. Animals treated with these cell combinations did respond to injections of turkey gamma globulin. These investigators (Chiller, et al., 1971) studied the kinetics of induction and spontaneous loss of tolerance in both bone marrow and thymic lymphocytes. At various times after tolerance

induction, tolerant bone marrow cells or tolerant thymus cells were transferred along with appropriate normal cells to lethally irradiated, syngeneic recipients. The anti-HGG response obtained in these animals after two challenges of heat aggregated HGG was compared with responses obtained in animals reconstituted with normal thymus and normal bone marrow cells. Thymus cells became 90 per cent unresponsive within 24 hrs of tolerogen administration and 100 per cent tolerant within 120 hrs. Thymocytes remained tolerant for a prolonged period before they returned to a responsive state by the 135th to 150th day. In contrast, tolerance in the bone marrow cell population did not begin to appear until 8 days after injection of sHGG, and it was not complete until the 21st day. Tolerance of the bone marrow cell population was not long lasting and by day 49 these cells had returned to normal responsiveness. Not only did thymus cells and bone marrow cells differ in regard to kinetics of tolerance induction, but they were distinctly different in their sensitivity to various doses of sHGG. Tolerance was induced in thymus cells with as little as 0.01 mg deaggregated HGG,whereas bone marrow cells did not become tolerant unless at least 100X more tolerogen was injected. Failures by other investigators to detect unresponsiveness in bone marrow cells could be explained by the fact that their assays of the immune potential of bone marrow cells from tolerant mice were carried out at a time when these cells would not be expected to be tolerant. Alternatively, these investigators may have used an insufficient amount of antigen for tolerance induction in the bone marrow cell population.

The discovery that thymus-dependent lymphocytes may be tolerant

in the unresponsive animal, while bone marrow-derived lymphocytes are not, helped explain previous observations that immune tolerance to albumins could be broken by injection of cross reacting albumins (Weigle, 1961; Weigle, 1965c; Benjamin and Weigle, 1970a, 1970b) or with albumins which had been chemically modified (Weigle, 1962; Paul, Thorbecke, Siskind, and Benacerraf, 1969). Paul and coworkers (1969) used DNP-BSA to terminate the unresponsive state of rabbits to BSA. Termination of tolerance could only be accomplished if the animals had been rendered tolerant by small but not large BSA doses. Normal levels of binding and precipitating antibodies to BSA were found by Benjamin and Weigle (1970a) in BSA-tolerant rabbits treated with cross reacting albumins. In addition, the antibody produced in the tolerant rabbits was qualitatively the same as that produced in normal animals. Benjamin and Weigle (1970b) observed that injections of BSA into BSA-tolerant rabbits which had been treated two months previously with cross reacting albumins resulted in the production of antibodies which were only directed against determinants shared by BSA and the albumin used for tolerance termination. If bone marrow cells were responsive in these animals, one would expect that thymus cells could react with determinants on the cross reacting albumin which were unrelated to those of BSA,while bone marrow cells could recognize determinants shared by BSA and the terminating albumin.

Chiller and Weigle (1972) determined the cellular requirement for rescue of immunocompetency of spleen cells of mice treated three days previously with deaggregated HGG. At this time period, it was expected that the T-cells in the spleen would be unresponsive,while B-cells

would be competent to respond to immunogenic HGG. Surprisingly, supplementation of tolerant spleen cells with normal thymus cells or normal bone marrow cells did not reverse the unresponsive state. When both normal bone marrow cells and normal thymus cells were added to tolerant spleen cells, immune responses were detected in reconstituted, x-irradiated recipients. These experiments suggested that peripheralized B-cells may become tolerant in a shorter period of time than do B-cells resident in the bone marrow. Such results agree with reported tolerance induction in vitro with thymus-independent antigens where unresponsiveness, presumably of B-lymphocytes, has been observed after brief exposure to antigen (Britton, 1969; Feldmann, 1971, 1972b; Diener and Armstrong, 1969; Feldmann and Diener, 1970).

The fate of cellular receptors for antigen in unresponsive mice was studied by Louis, Chiller and Weigle (1972). They determined the number of cells which could bind ^{125}I-HGG as time passed following injection of sHGG. There was a marked decrease in antigen binding cells (ABC) in the spleens of tolerogenized mice as early as 12 hrs after sHGG administration. Optimal reduction occurred at five days and remained at this level for at least 20 days. Antigen binding cells increased in mice immunized with aggregated-HGG beginning as early as 12 hrs after injection and reaching a peak at 3 days. Normal, immune and tolerant mice had equal numbers of ABC in the bone marrow until day 20 when there was a marked reduction of ABC in the bone marrow of tolerogen-treated mice. These results disagreed with the work of others (Humphrey and Keller, 1970; Ada et al., 1970; Sjoberg and Moller, 1970; Howard et al., 1969) who observed numbers of ABC in tolerant animals equal to or

greater than that observed in normal animals. Noar and Sulitzeneau (1967) and Katz, Davie, Paul and Benacerraf (1971) reported reduced numbers of ABC in tolerant animals. Differences in reported numbers of ABC in tolerant animals might be explained by the possibility that both bone marrow cells and thymus cells were not tolerant in animals where normal or increased numbers of ABC were observed. Additionally, investigators who have observed increased ABC in unresponsive animals have used antigens which are not efficient tolerogens (Weigle, Chiller, and Habicht, 1972).

It was proposed by Gershon and Kondo (1970) that tolerance induction in bone marrow cells may require the cooperation of thymus-dependent cells. They were unable to induce tolerance in thymus-dependent, bone marrow-derived cells unless thymocytes were present during the induction period. These workers (Gershon and Kondo, 1971) reported that tolerant spleen cells transferred along with normal thymus and bone marrow cells to irradiated recipients prevented the cooperative effect of the normal cells in an anti-sRBC response. Spleen cells from normal mice or from mice tolerogenized in the absence of thymus-derived cells did not effect cellular cooperation in the immune response to sheep erythrocytes. The ability of tolerant spleen cells to abolish immune responses was specific for the antigen to which the spleen cells were unresponsive. Gershon and Kondo suggested that tolerant thymus-dependent cells produce an immunosuppressive substance (IgY). Alternatively, unresponsive T-cells may influence bone marrow-derived cells to secrete this material. Chiller and Weigle (1972) could find no evidence that HGG-tolerant cells would inhibit responsiveness of normal

cells. Howard et al. (1972) determined that induction of tolerance to pneumococcal polysaccharide could be accomplished in bone marrow cells in the absence of thymus-dependent cells.

Statement of Problem

Golub and Weigle (1969) observed that various strains of inbred mice differ in their ability to be rendered immunologically tolerant with ultracentrifuged human gamma globulin (sHGG). The BALB/c strain which was highly refractory to the induction of the unresponsive state, and the C57BL/6 strain was highly susceptible to induction of tolerance with sHGG. The F_1 hybrid of a cross-mating between BALB/c and C57BL/6 mice behaved as its C57BL/6 parent in regard to its ease of tolerance induction. The present investigation was undertaken to evaluate the cellular basis (thymus cell and/or bone marrow cell) for the observed variation among these three strains of mice to induction of the unresponsive state.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

Male BALB/c, C57BL/6 and CBF_1 (BALB/c x C57BL/6) mice were purchased from Simenson Laboratories, Gilroy, California. The mice were seven weeks of age at the time of arrival and were used experimentally when they were 8 to 12 weeks old. In one experiment, female CBF_1 mice were employed. The animals were fed Tekland Mouse/Rat Diet and tap water ad libitum. Animals used as irradiated recipients of syngeneic cells were maintained for one to two weeks prior to irradiation on water which contained a 1:4000 dilution of chlorox. After irradiation and cell transfer animals were housed no more than three to a cage and were continued on chlorinated water for the duration of the experiment.

Goats used for preparation of amplifying antiserum were obtained locally. They were fed a diet of Purina meal (#2535M) and water ad libitum.

Antigens

Human gamma globulin (HGG, Cohn Fraction II) in lyophilized form was generously supplied by the American Red Cross. Various preparations of this material were used for tolerance induction, antigenic challenge and assay of the immune response.

Soluble Human Gamma Globulin

An aggregate-free preparation of human gamma globulin, designated soluble human gamma globulin (sHGG), was used for tolerance induction. Lyophilized HGG was dissolved in physiological saline solution (0.85 per cent NaCl) at room temperature. The solution was centrifuged for 150 min using a swinging bucket rotor (SW41) at 100,000 X G, 4 C, in a Beckman Model L2-65B preparative ultracentrifuge. After centrifugation the upper one third of the material in each cup was carefully removed, taking care to avoid agitation of the protein solution. The protein concentration of the solution was determined spectrophotometrically by the method of Kalckar (1947) using a Perkin-Elmer double beam spectrophotometer (Coleman 124). Animals were injected with a volume of the sHGG solution sufficient to contain the amount of tolerogen desired. The volume injected was approximately 0.5 ml. All animals were injected within 20 to 30 min following centrifugation of the protein solution.

Aggregated Human Gamma Globulin

For immunogenic challenge, a heat aggregated form of HGG (agg-HGG) was prepared according to the method of Biro and Garcia (1965). Forty milliliters of a saline (0.85 per cent NaCl) solution of HGG containing 25 mg protein per ml was dialyzed in the cold (4 C) for 24 hrs against two changes of 0.01 M phosphate buffer (pH 8.0) made up in distilled, deionized water. The buffer contained 0.49 gm KH_2PO_4 and 11.23 gm $NaHPO_4$ in 6 l distilled water. After dialysis the HGG solution was centrifuged in an International clinical centrifuge to remove any precipitate. The supernatant fluid was removed and was heated at 63 C for 20 min with occasional stirring. Timing of heat treatment was begun

when the solution in the flask reached 63 C. After heating, the protein solution was cooled in an ice bath for 2 hrs. The aggregated material was collected by centrifugation at 1000 X G for 15 min in an International PR-1 refrigerated centrifuge and was washed three times in phosphate buffer. After the final wash the aggHGG was resuspended in 20 ml saline. One milliliter of aggHGG was digested in 4.0 ml 0.01 N NaOH for thirty min. The protein concentration of the digest was determined spectrophotometrically (Kalckar, 1947). The original suspension of aggHGG was diluted with saline to contain 2.0 mg protein per ml and was frozen (-20 C) in 5 ml aliquots until used. Aggregated antigen was used within 45 days of its preparation.

Preparation of Amplifying Antiserum

Goat anti-mouse globulin used to develop indirect plaque forming cells in the localized hemolysis-in-gel assay was prepared as follows. Goats were given three injections (subcutaneously in four sites) of 10 mg mouse gamma globulin (Cohn Fraction II, Pentex, Inc., Kankakee, Illinois, lot #9) suspended in 5 ml incomplete Freund's adjuvant. The injections were spaced three weeks apart. Three weeks after the final injection the goats were bled and their serum tested against a control for its ability to detect antibody forming cells in the spleens of mice undergoing a secondary response to aggHGG. Amplifying serum kindly supplied by Dr. Jacques Chiller (Scripps Clinic and Research Foundation, La Jolla, California) was used as the reference control. This serum was also used experimentally in some cases.

Immune Response Assay

The immune response of experimental animals was evaluated by the localized hemolysis-in-gel assay of Jerne, Nordin and Henry (1963) as modified by Golub et al. (1968). Goat erythrocytes (gRBC) preserved in Alsever's solution (Animal Blood Center, Syracuse, N. Y.) were aged upon receipt for four weeks. The red blood cells were washed three times in conjugation buffer (CB) containing 4.35 gm NaCl, 2.4 gm KH_2PO_4 and 10.50 gm Na_2HPO_4 per 1 distilled, deionized water. The pH of the buffer was adjusted to 7.2 using the appropriate 0.15 M salt.

A soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl, ECDI, Ott Chemical Co., Muskegon, Michigan) was used to couple human gamma globulin to gRBC. Four tenths ml of a 50 per cent erythrocyte suspension in CB were added to 3 ml of a 20 mg per ml solution of HGG in CB. The HGG solution had been previously absorbed with fresh gRBC. Five tenths ml of an ECDI solution in CB containing 100 mg per ml was then added to the erythrocyte-protein mixture. The ECDI solution was made up immediately before addition to the reaction mixture. The suspension was incubated at 4 C for 1 hr and was centrifuged at 1,500 rpm for 5 min. The red cell pellet was washed two times in CB and once in Hank's balanced salts solution (HBSS, Microbiological Associates, Bethesda, Maryland). The erythrocytes were suspended in HBSS to a final concentration of 7 per cent. Control cells were prepared in a manner similar to the HGG-conjugated cells except 3 ml of CB were substituted for the HGG solution. In some cases coupled erythrocytes were used as many as three days after conjugation; in these instances the red blood cells were washed 1X in HBSS on the day of use and were resuspended in

HBSS.

Animals were sacrificed by cervical dislocation and their spleens removed for preparation of spleen cell suspensions. Single cells were obtained by pressing spleens with a pair of forceps through a 60 mesh wire screen into cold HBSS. The cells were filtered through #80 mesh silk screen into a tube (15 x 100 mm). The spleen cell suspensions were centrifuged for 10 min at 1,000 X G in an International refrigerated centrifuge (PR-1) at 4 C. The pellet was resuspended in 5 ml 0.83 per cent NH_4Cl and allowed to stand at room temperature for 5 min to lyse the red cells present in the preparation. The cells were again spun down and were washed three times in cold HBSS. After the final wash the cells were resuspended in 2.0 ml HBSS, and the cell concentration was determined using a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida). Samples of the spleen cell suspension or appropriate dilutions of the spleen cell suspension were mixed in a small tube (10 x 75 mm) with 0.05 ml erythrocyte suspension and 0.4 ml of a 0.5 per cent solution of agarose (Mann Research Laboratories, Inc., Orangeburg, New York) in HBSS. The agarose solution had been previously melted in the autoclave and was held in a water bath at 48 C. After addition of the agarose the contents of the tube were mixed on a vortex mixer and poured over the surface of a 1" x 3" glass microscope slide. Microscope slides were precoated with a 0.1 per cent solution of agarose in distilled water and were allowed to dry before use. After the erythrocyte-spleen cell-agarose mixture had solidified on the microscope slide, the slides were inverted on a specially designed tray over a solution of HBSS containing 10 per cent guinea pig complement (Colorado

Serum Co., Denver, Colorado) and a 1:100 dilution of goat anti-mouse globulin. The trays were incubated in a humid chamber at 37 C for three hours. Indirect plaques were enumerated using a small grid and a 5X hand lens with direct light through a blue filter. The number of plaques was expressed as plaque forming cells per 1 x 10^6 nucleated spleen cells. Each assay was carried out in duplicate. The average number of PFC in the duplicate determinations was corrected for background plaques to goat erythrocytes as determined by counting the number of PFC on slides where spleen cells were plated with control red blood cells.

Determination of Dose of sHGG for Tolerance in Intact Animals

Groups of 8-9 animals of each of the three strains of mice under investigation were injected intraperitoneally with 0.5 mg, 2.5 mg, or 12.5 mg sHGG. Ten days later the animals were challenged with an intraperitoneal (IP) injection of 0.6 mg aggHGG. The animals received a secondary challenge of 0.6 mg aggHGG by the IP route on day 20. Four days later they were sacrificed and suspensions of their spleen cells assayed for indirect plaque forming cells to HGG. Animals which had been treated with saline instead of sHGG served as immunization controls.

Cell Transfer Experiments

X-irradiation

In order to determine the dose of x-irradiation to use in cell transfer experiments, an irradiation death experiment was performed with each mouse strain. Animals were subjected to various doses of irradiation (350-950 Rads) with a Theratron 80 ^{60}Cobalt source (Atomic Energy of Canada, Ltd.) delivering 145.77 rads per min. The source was

80 cm from the midline and the field was 20 cm x 20 cm. After treatment the animals were housed three to a cage and were observed daily for deaths over a period of thirty days. The dose which resulted in 60 per cent mortality at 30 days was choosen to be used for treatment of recipients in cell transfer experiments.

Preparation of Cells for Transfer

Mice were sacrificed by decapitation which allowed exsanguination so that the thymus could be removed free of blood. The thymus was removed from the thoracic cavity taking care to detach all adjacent lymph nodes. Single thymocytes were obtained by pressing a pool of donor thymi through a 60 mesh wire screen into cold HBSS which contained 100 units penicillin and 100 μg streptomycin per ml. HBSS containing antibiotics was used as the suspending fluid throughout the transfer experiment. The cells were filtered through #80 silk and were washed three times in a refrigerated centrifuge. After the final wash the thymocytes were resuspended in HBSS and were again filtered through silk screen.

Bone marrow cells were obtained by applying hydrostatic pressure to the femours and tibiae using a 23 ga needle attached to a syringe filled with HBSS. The cells were dispersed by first repeatedly flushing the bone marrow-balanced salts solution out of a syringe without a needle. Further cell dispersion was accomplished by attaching an 18 ga needle to the syringe and repeating the flushing process. The bone marrow cells were filtered through silk screen and washed three times in cold HBSS. The supernatant fluid of each wash was carefully aspirated from the top so that all lipids would be removed during the washing

process. Cells were resuspended in HBSS and filtered through silk screen for a second time to remove any large clumps.

Spleen cells were prepared in a manner similar to that described for the localized hemolysis-in-gel assay except that they were not treated with ammonium chloride to lyse red blood cells.

Cell concentrations were determined using a Coulter Counter (Model B). Any red cells present in the cell suspensions were lysed with Zap-Isoton (Coulter Electronics) before the cells were counted.

Determination of Dose of sHGG Required to Render Thymus Cells and Bone Marrow Cells Unresponsive

Induction of immunologic unresponsiveness at the thymus cell and bone marrow cell levels was evaluated in a manner similar to that described by Chiller, Habicht, and Weigle (1970). An outline of the experimental protocol appears in Table 1. Donor animals were treated with tolerogenic sHGG (0.5 mg, 2.5 mg, or 12.5 mg) or saline by intraperitoneal injection. Ten or twenty-two days later the donors were sacrificed and suspensions of bone marrow and thymus cells were used to reconstitute lethally irradiated, syngeneic recipients. Tolerance of bone marrow cells was assayed by transferring bone marrow cells from tolerant donors together with thymocytes derived from saline treated, normal donors. In like manner, transfer of tolerant thymocytes plus normal bone marrow cells was used to indicate whether thymus cells had become tolerant by the sHGG treatment. Animals reconstituted with normal thymocytes and normal bone marrow cells served as immunization controls.

After preparation of cells as previously described, each

TABLE 1

EXPERIMENTAL DESIGN FOR DETERMINATION OF BONE MARROW AND THYMUS CELL TOLERANCE

Day	Treatment	Experimental		Control
1	X-irradiation	+	+	+
	Cell transfer:[a] Thymus Bone Marrow	Normal Tolerant	Tolerant Normal	Normal Normal
	AggHGG[b]	+	+	+
11	AggHGG	+	+	+
16	Jerne plaque assay	+	+	+

[a]Donors of tolerant cells were treated ten or twenty-two days previously with 0.5 mg, 2.5 mg, or 12.5 mg soluble human gamma globulin.

[b]Heat aggregated human gamma globulin.

irradiated recipient was given an intravenous injection of appropriate mixtures of 15 x 10^6 bone marrow cells and 45 x 10^6 thymus cells. Animals were reconstituted 4 to 6 hrs after irradiation. Care was taken to carry out the entire procedure of cell preparation and transfer within 3 hrs. On the same day as the cell transfer,each recipient was challenged intraperitonealy with 0.6 mg aggHGG, and ten days later each animal was given a similar aggHGG injection. Five days after the secondary antigenic challenge, the animals were sacrificed and their spleens assayed for indirect PFC to human gamma globulin. Determinations of tolerance in thymus cells were made at 10 days after tolerance induction, while bone marrow cell unresponsiveness was evaluated at 10 and 22 days after sHGG treatment.

Determination of Effect of Tolerant Thymus Cells on the Responsiveness of Normal Cells

Lethally irradiated CBF_1 male mice were treated with an IV injection of mixtures of tolerant and normal cells or with mixtures of normal cells and cells taken from donors x-irradiated on the day of cell transfer. Tolerant donors were treated 10 days previously with 5.0 mg sHGG. Experimental groups consisted of animals (10 each) treated with a) 45 x 10^6 normal thymus cells, 45 x 10^6 tolerant thymus cells, and 15 x 10^6 normal bone marrow cells, b) 45 x 10^6 normal thymus cells, 45 x 10^6 irradiated thymus cells, and 15 x 10^6 normal bone marrow cells, and c) 45 x 10^6 tolerant thymus cells and 15 x 10^6 normal bone marrow cells. The experimental protocol is outlined in Table 2. Normal and tolerant thymus cells were administered on the first day. The next day all animals received an injection of bone marrow cells and an IP

TABLE 2

EXPERIMENTAL DESIGN FOR ASSAY OF THE EFFECT OF TOLERANT THYMUS CELLS ON THE RESPONSIVENESS OF NORMAL CELLS

Day	Treatment	Experimental	Control	
			Immune	Tolerant
1	X-irradiation	+	+	+
	Cell transfer:[a] Thymus	Normal + Tolerant	Normal + Irradiated	Tolerant
2	Cell transfer: Bone marrow	Normal	Normal	Normal
	AggHGG[b]	+	+	+
12	AggHGG	+	+	+
17	Jerne plaque assay	+	+	+

[a]Donors of tolerant cells were treated ten days previously with 5.0 mg soluble human gamma globulin. X-irradiated thymus cells were obtained from donors lethally irradiated (950 R) on the day of sacrifice.

[b]Heat aggregated human gamma globulin.

injection of 0.6 mg aggHGG. A secondary challenge with 0.6 mg aggHGG was administered 10 days later, and after an additional 5 days animals were assayed for indirect PFC against HGG by the localized hemolysis-in-gel test.

Determination of Effect of Tolerant Spleen Cells on the Responsiveness of Normal Cells

Spleen cells taken from donors treated 10 days previously with 5.0 mg sHGG were administered to irradiated, syngeneic CBF_1 mice to test whether these cells would affect the responsiveness of normal cell combinations. The experimental groups consisted of irradiated animals treated with an IV injection of a) normal thymocytes (45×10^6) and tolerant spleen cells (1×10^8), b) normal thymocytes (45×10^6) and normal spleen cells (1×10^8), c) normal thymocytes (45×10^6) and spleen cells if irradiated (950 R) donors (1×10^8), and d) tolerant spleen cells (1×10^8). The experimental protocol is presented in Table 3. The day following the administration of the above cell combinations, all animals were given an IV injection of 15×10^6 normal bone marrow cells. At the same time 0.6 mg aggHGG was administered by the IP route. A second IP injection of 0.6 mg aggHGG was given 10 days later, and 5 days following this challenge, animals were sacrificed and the number of anti-HGG PFC in suspensions of their spleen cells was determined.

TABLE 3

EXPERIMENTAL DESIGN FOR ASSAY OF THE EFFECT OF TOLERANT SPLEEN CELLS ON THE RESPONSIVENESS OF NORMAL CELLS

Day	Treatment	Experimental	Control		
			Immune		Tolerant
1	X-irradiation	+	+	+	+
	Cell transfer:[a] Spleen Thymus	Tolerant Normal	Normal Normal	Irradiated Normal	Tolerant -
2	Cell transfer: Bone marrow	Normal	Normal	Normal	Normal
	AggHGG[b]	+	+	+	+
12	AggHGG	+	+	+	+
17	Jerne plaque assay	+	+	+	+

[a]Donors of tolerant cells were treated ten days previously with 5.0 mg soluble human gamma globulin. X-irradiated spleen cells were obtained from donors lethally irradiated (950 R) on the day of sacrifice.

[b]Heat aggregated human gamma globulin.

CHAPTER III

RESULTS

Tolerance Induction in Intact Animals

For determination of the susceptibility of BALB/c, C57BL/6, and CBF_1 mice to tolerance induction, groups of animals were treated with three doses of soluble human gamma globulin (tolerogen), and were challenged later with 2 injections of heat aggregated HGG (aggHGG). The animals were assayed by the localized hemolysis-in-gel technique for indirect plaque forming cells (PFC) to human gamma globulin. The mean PFC response in each experimental group was used to determine the per cent suppression according to the following formula:

$$\text{Per cent suppression} = \frac{\text{Mean PFC response of immunization controls minus mean PFC response of experimental group}}{\text{Mean PFC response of immunization controls}} \times 100$$

The results of the experiment are shown in Figure 1. BALB/c mice were relatively difficult to render unresponsive to human gamma globulin. Doses of 0.5 mg and 2.5 mg tolerogen resulted in 44 per cent and 23 per cent suppression of the anti-HGG PFC response, respectively. However, anti-HGG responses of BALB/c mice were suppressed 97.5 per cent by treatment with 12.5 mg soluble human gamma globulin (sHGG).

C57BL/6 mice were highly susceptible to tolerance induction with sHGG. These animals were 98 per cent unresponsive after treatment

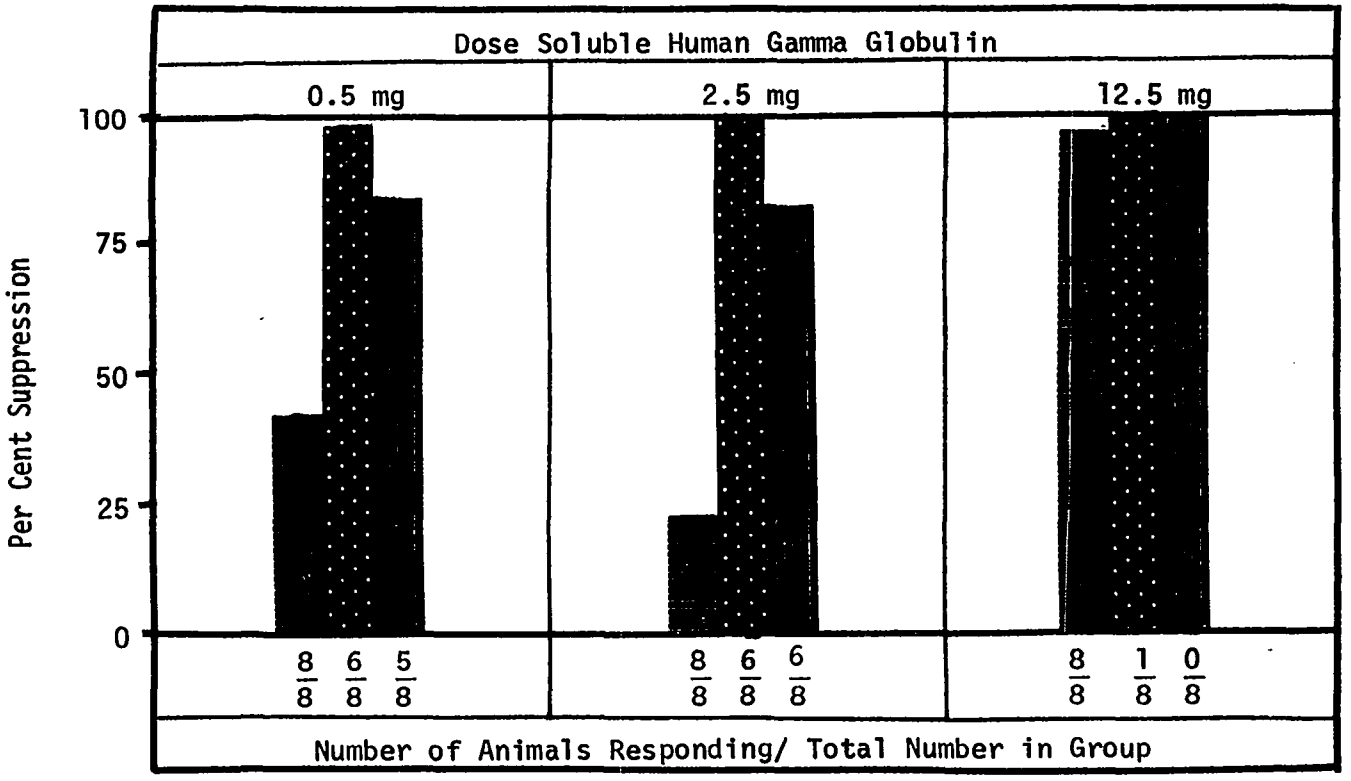

Figure 1. Per cent suppression of anti-human gamma globulin plaque forming cell response of intact animals treated 10 days previously with various doses of soluble human gamma globulin. Number of immunization controls responding/ total number in group: BALB/c = 9/9, C57BL/6 = 9/9, CBF_1 = 9/9. Horizontally striped bars - BALB/c, dotted bars - C57BL/6, vertically striped bars - CBF_1.

with as little as 0.5 mg tolerogen. At the higher doses of sHGG (2.5 mg and 12.5 mg) C57BL/6 mice were 100 per cent unresponsive.

CBF_1 mice, the F_1 hybrid of BALB/c and C57BL/6 mice, were similar to their C57BL/6 parent in regard to their ease of tolerance induction. The anti-HGG response in animals treated with 0.5 mg sHGG showed an 87 per cent suppression of the normal response. Doses of 2.5 mg and 12.5 mg SHGG resulted in 86 and 100 per cent suppression, respectively, of the immune response to HGG.

Although BALB/c mice could be made tolerant by 12.5 mg soluble human gamma globulin, none of the animals were totally unresponsive. All animals in this group had low anti-HGG PFC responses. On the other hand, 1 of 8 C57BL/6 and 0 of 8 CBF_1 mice had a residual anti-HGG response when treated with 12.5 mg sHGG. Seventy-five per cent of C57BL/6 mice treated with 0.5 mg or 2.5 mg sHGG were still responsive. Treatment of CBF_1 mice with 0.5 mg tolerogen failed to induce complete suppression in 62.5 per cent of the animals while at the 2.5 mg sHGG dose 37.5 per cent of the animals were responsive.

Determination of Irradiation Dose for Cell Transfer Experiments

Groups of mice were exposed to various doses of x-irradiation ranging from 350 R to 950 R, and the number of animals surviving were enumerated daily (representative data are found in Tables 4, 5 and 6). The dose of irradiation which resulted in death of approximately 60 per cent of the mice thirty days after treatment was determined by plotting the percentage of animals surviving at 30 days ($LD_{60/30}$) against the irradiation dose on a semi-logarithmic scale. This dose was used in

TABLE 4

SURVIVAL OF BALB/c MICE AFTER EXPOSURE TO VARIOUS DOSES OF X-IRRADIATION

Dose (Rads)	Day After Irradiation[a]					
	5	10	15	20	25	30
350	9/9	9/9	9/9	9/9	9/9	9/9
450	10/10	10/10	9/10	8/10	8/10	8/10
550	10/10	10/10	8/10	7/10	7/10	6/10
650	10/10	10/10	8/10	3/10	2/10	2/10
750	9/9	9/9	4/9	2/9	1/9	1/9
850	9/9	9/9	1/9	0/9	0/9	0/9

[a]Number of survivors/total number in group.

TABLE 5

SURVIVAL OF C57BL/6 MICE AFTER EXPOSURE TO VARIOUS DOSES OF X-IRRADIATION

Dose (Rads)	Day After Irradiation[a]					
	5	10	15	20	25	30
350	6/6	6/6	6/6	6/6	6/6	6/6
450	6/6	6/6	6/6	6/6	6/6	6/6
550	6/6	6/6	5/6	5/6	5/6	5/6
650	9/9	9/9	9/9	9/9	9/9	9/9
750	9/9	7/9	5/9	5/9	4/9	4/9
850	9/9	8/9	7/9	7/9	7/9	7/9
950	8/9	0/9	0/9	0/9	0/9	0/9

[a]Number of survivors/total number in group.

TABLE 6

SURVIVAL OF CBF_1 MICE AFTER EXPOSURE TO VARIOUS DOSES OF X-IRRADIATION

Dose (Rads)	Day After Irradiation[a]					
	5	10	15	20	25	30
650	9/9	9/9	9/9	9/9	9/9	9/9
750	9/9	9/9	9/9	9/9	9/9	9/9
850	9/9	9/9	9/9	8/9	8/9	8/9
950	9/9	8/9	4/9	4/9	4/9	4/9

[a]Number of survivors/total number in group.

subsequent cell transfer experiments in which recipient animals were x-irradiated. The $LD_{60/30}$ dose was chosen to avoid early deaths which occur at higher irradiation doses due to destruction of the intestinal epithelium. The $LD_{60/30}$ for BALB/c mice was 600 R. C57BL/6 mice used as recipients of transferred cells were irradiated with 750 R, while CBF_1 mice were treated with 950 R.

Induction of Tolerance in Bone Marrow and Thymus Cells

Ten Days Post Tolerance Induction

After in vivo exposure of thymus and bone marrow cells to sHGG for a period of ten days, these cells were transferred along with the appropriate normal cell type (normal thymus cells + tolerant bone marrow cells or tolerant thymus cells + normal bone marrow cells) to x-irradiated, syngeneic recipients to determine their immune status. Spleen cell suspensions of recipient animals were assayed for anti-HGG PFC following two challenges with aggHGG. The per cent suppression caused by sHGG treatment was calculated as previously described. Results are listed in Figures 2 and 3 and in Table 7.

Thymocytes taken from BALB/c mice which had been injected with 0.5 mg tolerogen ten days previously were completely responsive (Figure 2). Thymus cells of BALB/c mice were 14 per cent unresponsive after exposure to 2.5 mg sHGG, whereas 12.5 mg sHGG resulted in a 99 per cent suppression of the thymus cell population in the BALB/c mouse strain.

In contrast to results obtained in the BALB/c strain, C57BL/6 thymocytes were extremely sensitive to tolerance induction. C57BL/6 thymus cells were suppressed 90 per cent by 0.5 mg sHGG. When 2.5 mg

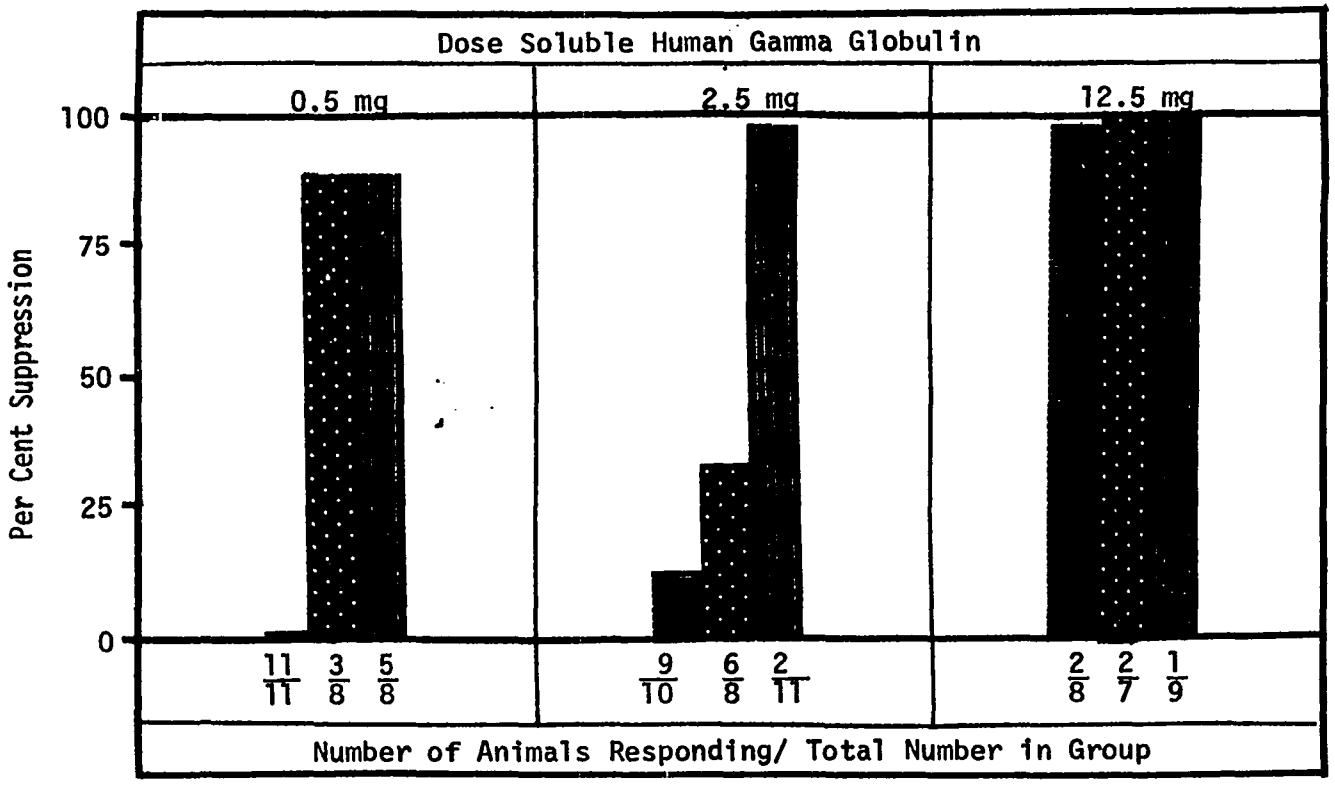

Figure 2. Per cent suppression of anti-human gamma globulin plaque forming cell response of irradiated animals reconstituted with normal bone marrow cells and thymus cells taken from donors treated 10 days previously with soluble human gamma globulin. Number of animals reconstituted with normal bone marrow and thymus cells responding/ total number in group: BALB/c = 26/28, C57BL/6 = 16/20, CBF_1 = 22/24. Horizontally striped bars - BALB/c, dotted bars - C57BL/6, vertically stripped bars - CBF_1.

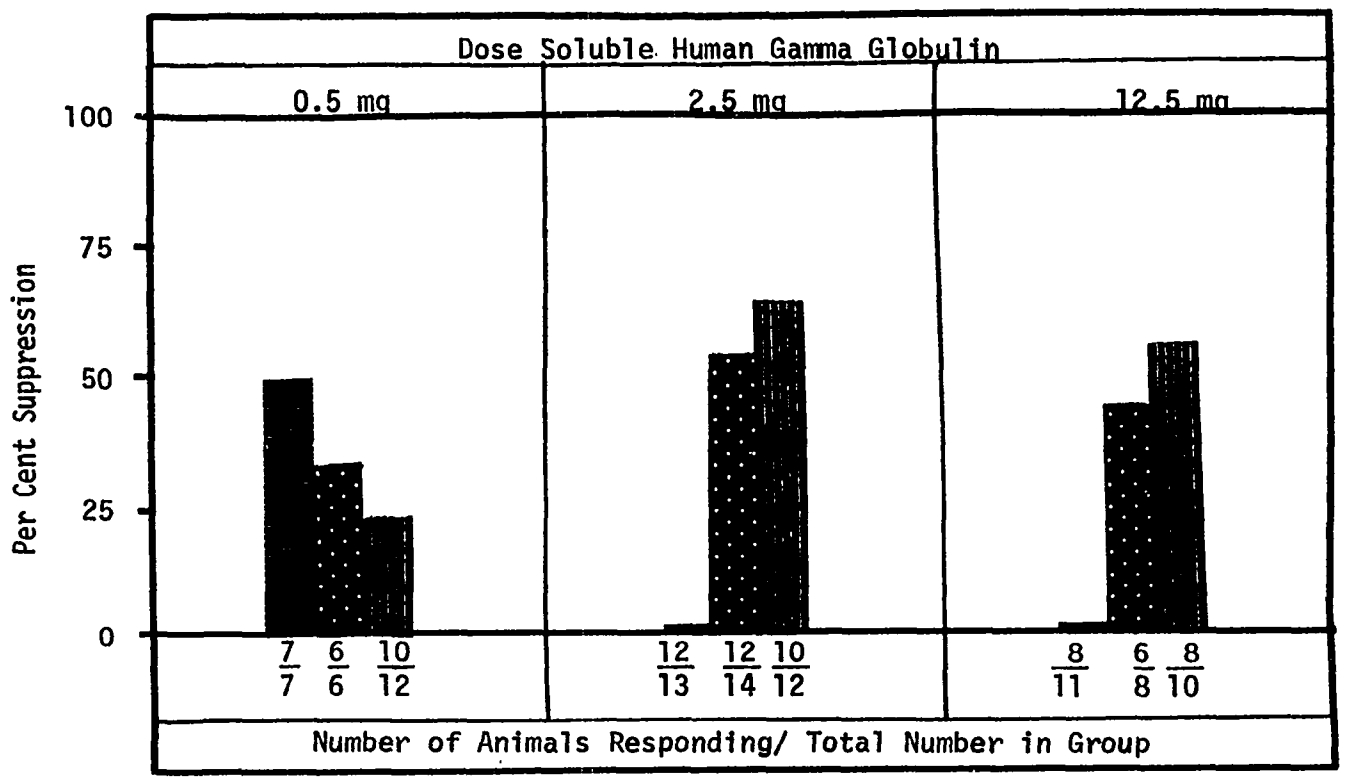

Figure 3. Per cent suppression of anti-human gamma globulin plaque forming cell response of irradiated animals reconstituted with normal thymus cells and bone marrow cells taken from donors treated 10 days previously with soluble human gamma globulin. Number of animals reconstituted with normal bone marrow and thymus cells responding/ total number in group: BALB/c = 26/28, C57BL/6 = 16/20, CBF_1 = 22/24. Horizontally striped bars - BALB/c, dotted bars - C57BL/6, vertically striped bars - CBF_1.

TABLE 7

EFFECT OF TREATMENT OF CELL DONORS WITH VARIOUS DOSES OF SOLUBLE HUMAN GAMMA GLOBULIN ON THE ABILITY OF THYMUS AND BONE MARROW CELLS TO RECONSTITUTE IRRADIATED RECIPIENTS: A COMPILATION OF THREE EXPERIMENTS

Mouse Strain	Number of Animals	Cells Transferred[a]	Dose Tolerogen (mg)	PFC/10^6 Spleen Cells[b]	Range
BALB/c	29	NT-NBM	-	11.2	0 - 103.0
	11	TT-NBM	0.5	16.8	0.4 - 131.4
	10		2.5	12.2	0 - 56.0
	8		12.5	0.1	0 - 0.5
	7	NT-TBM	0.5	4.1	0.3 - 9.0
	13		2.5	30.0	0 - 250.0
	11		12.5	13.0	0 - 74.0
C57BL/6	20	NT-NBM	-	12.7	0 - 58.8
	8	TT-NBM	0.5	1.7	0 - 8.9
	8		2.5	5.5	0 - 36.8
	7		12.5	0.2	0 - 1.1
	12	NT-TBM	0.5	4.9	1 - 12.2
	14		2.5	7.3	0 - 20.8
	8		12.5	5.9	0 - 17.5
CBF_1	24	NT-NBM	-	11.7	0 - 35.7
	9	TT-NBM	0.5	2.4	0 - 11.3
	11		2.5	0.1	0 - 0.9
	9		12.5	0.1	0 - 0.5
	12	NT-TBM	0.5	9.5	0 - 27.3
	12		2.5	5.0	0 - 28.3
	10		12.5	5.7	0 - 30.7

[a]NT - normal thymus cells; TT - tolerant thymus cells; NBM - normal bone marrow cells; TBM - tolerant bone marrow cells. Tolerant donors were treated 10 days previously with soluble human gamma globulin.

[b]Number of anti-human gamma globulin plaque forming cells per 1×10^6 nucleated spleen cells.

tolerogen was administered, C57BL/6 thymus cells were 36.6 per cent unresponsive. It is possible that 2.5 mg tolerogen actually suppressed C57BL/6 thymus cells to a greater extent than these results indicate as one animal in this group had an extremely high PFC response (36.8/ 10^6 spleen cells). The remainder of the group had a mean PFC of 0.8/ 10^6 spleen cells. When the per cent suppression was calculated excluding this high responder, thymus cells in the C57BL/6 strain of mice were found to be 97 per cent suppressed by 2.5 mg tolerogen. When 12.5 mg sHGG was given, C57BL/6 thymus cells were 98.6 per cent tolerant.

Thymus cells of CBF_1 mice became tolerant as easily as did those of their C57BL/6 parent. Five tenths milligram of sHGG resulted in 90 per cent suppression of this cell population. CBF_1 thymocytes were 98 per cent tolerant after treatment with 2.5 mg sHGG, and at the 12.5 sHGG dose, CBF_1 thymus cells were 100 per cent suppressed.

Tolerance in bone marrow cells after ten days exposure to sHGG was more difficult to accomplish (Figure 3 and Table 7). BALB/c bone marrow cells were 55.3 per cent suppressed by 0.5 mg sHGG, however at higher doses of tolerogen (2.5 mg and 12.5 mg) no tolerance was detected in bone marrow cells from this strain. Bone marrow cells taken from C57BL/6 mice were 37 per cent unresponsive at the 0.5 mg sHGG dose. After treatment with 2.5 mg or 12.5 mg tolerogen, these cells were suppressed 57.7 and 48.6 per cent, respectively. CBF_1 bone marrow cells were likewise more refractory to tolerance induction than were thymus cells. A dose of 0.5 mg sHGG suppressed the responsiveness of these cells to 23 per cent, whereas 2.5 mg sHGG resulted in 68 per cent tolerance in this cell population. Injection of CBF_1 mice with 12.5 mg

tolerogen resulted in bone marrow cells which exhibited 59.5 per cent suppression of their anti-HGG PFC response.

Bone marrow cells in each of the three strains of mice were only rarely, if ever, completely unresponsive. In those experimental groups where some animals were totally unresponsive, comparison of the percentage of animals in that group which were responsive to the percentage of responsive animals which had been grafted with normal bone marrow and thymus cells showed no significant differences. The percentage of animals reconstituted with normal thymus cells plus normal bone marrow cells which failed to respond could be as high as 20 per cent.

Twenty-Two Days Post Tolerance Induction

An experiment was performed to assay suppression of bone marrow cell populations of BALB/c, C57BL/6, and CBF_1 mice 22 days after injection of sHGG as Chiller et al. (1971) had reported that tolerance in bone marrow cells of strain A/J mice was not complete until 22 days following tolerogen administration. Results of this experiment are shown in Figure 4. Twenty-two days after injection of 0.5 mg or 2.5 mg sHGG, BALB/c bone marrow cells were 67 and 45 per cent tolerant, respectively. When 12.5 mg tolerogen was administered BALB/c bone marrow cells were completely responsive. One animal reconstituted with normal thymus cells and normal bone marrow cells responded with 111 PFC per 10^6 spleen cells while the mean PFC response of the rest of the animals in the group was 21.5 PFC per 10^6 spleen cells. Calculation of the data excluding this high responder showed that BALB/c bone marrow cells were suppressed 47, 12 and 0 per cent by 0.5 mg, 2.5 mg and 12.5 mg tolerogen,

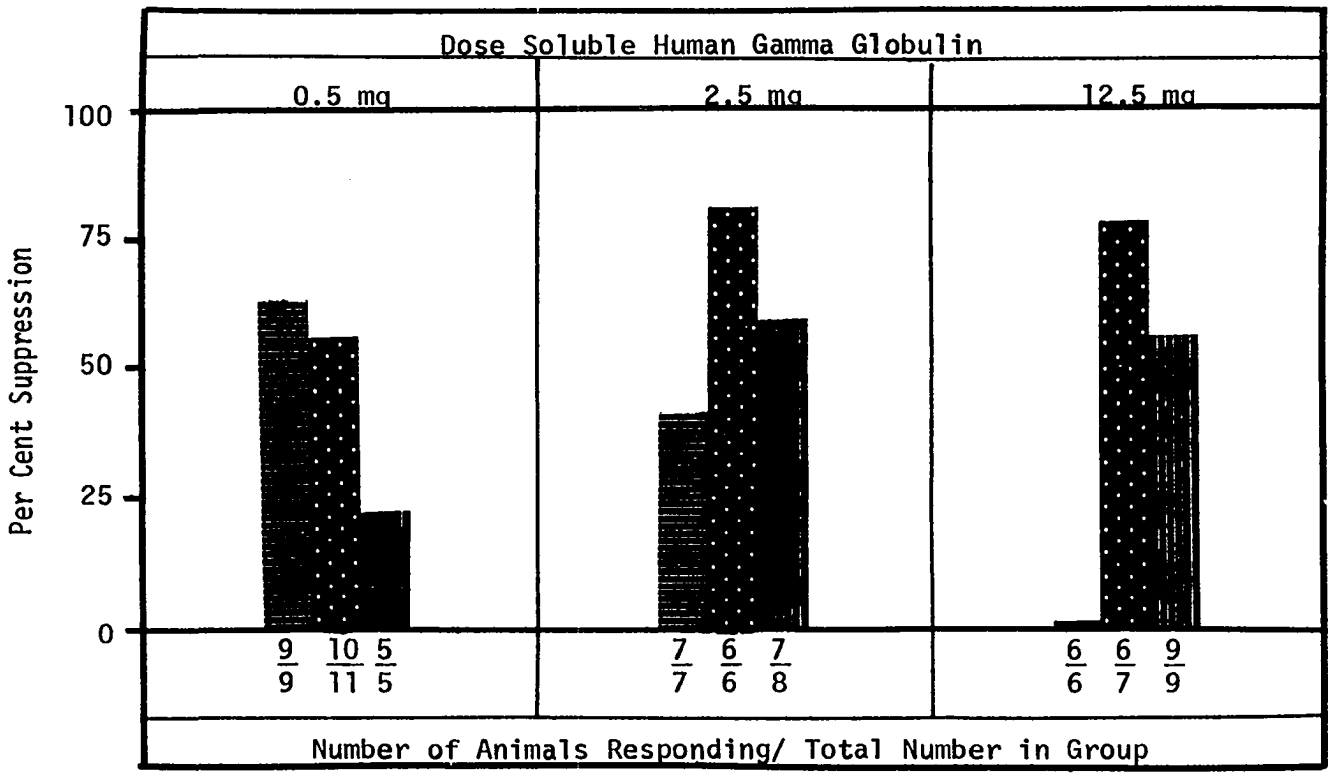

Figure 4. Per cent suppression of anti-human gamma globulin plaque forming cell response of irradiated animals reconstituted with normal thymus cells and bone marrow cells taken from donors treated 22 days previously with soluble human gamma globulin. Number of animals reconstituted with normal bone marrow and thymus cells responding/ total number in group: BALB/c = 28/28, C57BL/6 = 16/20, CBF_1 = 22/24. Horizontally striped bars - BALB/c, dotted bars - C57BL/6, vertically striped bars - CBF_1.

respectively. Bone marrow cells of C57BL/6 mice were 59 per cent tolerant 22 days after administration of 0.5 mg sHGG. Unresponsiveness of the bone marrow cell population increased to 85 per cent when the animals were treated with 2.5 mg sHGG. These cells were suppressed 82 per cent by 12.5 mg tolerogen. CBF_1 bone marrow cells were suppressed 35 per cent 22 days after injection of 0.5 mg sHGG. Administration of 2.5 mg or 12.5 mg tolerogen resulted in 63 and 60 per cent tolerance, respectively.

As was the case of the experiment in which bone marrow cells were exposed to tolerogen for 10 days, bone marrow cells exposed to sHGG for 22 days were hyporesponsive rather than totally unresponsive. However, the number of responsive animals in experimental groups was not significantly different from the number of responsive animals in control groups.

These results indicated that bone marrow cells could develop a higher degree of unresponsiveness if the duration of tolerogen exposure was increased. C57BL/6 bone marrow cells were rendered unresponsive more easily than were BALB/c bone marrow cells. In fact, tolerance comparable to that achieved in the C57BL/6 bone marrow cells could not be attained in BALB/c bone marrow cells even at high doses of sHGG. Although CBF_1 bone marrow cells did not become as unresponsive as did those of their C57BL/6 parent, cells from both strains reached their maximum degree of unresponsiveness at an sHGG dose of 2.5 mg. It could be said, therefore, that CBF_1 bone marrow cells became tolerant in a manner similar to that of their C57BL/6 parent.

Effect of Sex on Susceptibility of CBF_1 Mice to Tolerance Induction

CBF_1 mice are the progeny of the mating of a BALB/c female with a C57BL/6 male. Because it was apparent that the CBF_1 had inherited the high susceptibility to induction of tolerance of its C57BL/6 father, and because male CBF_1 mice had been used in previous experiments, it was of interest to determine if this trait was inherited as a Y-linked characteristic. Female CBF_1 mice were treated with three doses of sHGG, and ten days later thymus cells were transferred, along with normal bone marrow cells, to x-irradiated CBF_1 female recipients. Following two challenges with heat aggregated human gamma globulin, the PFC response of individual mice was determined, and the per cent suppression calculated. For comparative purposes data obtained in separate experiments utilizing CBF_1 male mice were included with the data from the experiment with CBF_1 females (Figure 5).

Thymus cells of CBF_1 female mice did not become unresponsive following treatment with 0.5 mg sHGG, whereas at this same tolerogen dose CBF_1 male thymus cells were 90 per cent tolerant. When 2.5 mg sHGG was administered CBF_1 female and CBF_1 male thymocytes had reached comparable levels of suppression (100 and 98 per cent, respectively). Both male and female thymus cells were fully suppressed at the 12.5 mg sHGG dose as well. Although tolerance induction was more difficult to achieve in the CBF_1 female than in the male, the female did not inherit the very high resistance to tolerance induction characteristic of its BALB/c mother. From these results it appeared that the Y-chromosome alone was not responsible for inheritance of this very high susceptibility to tolerance induction. Apparently the sex of the animal dose have some

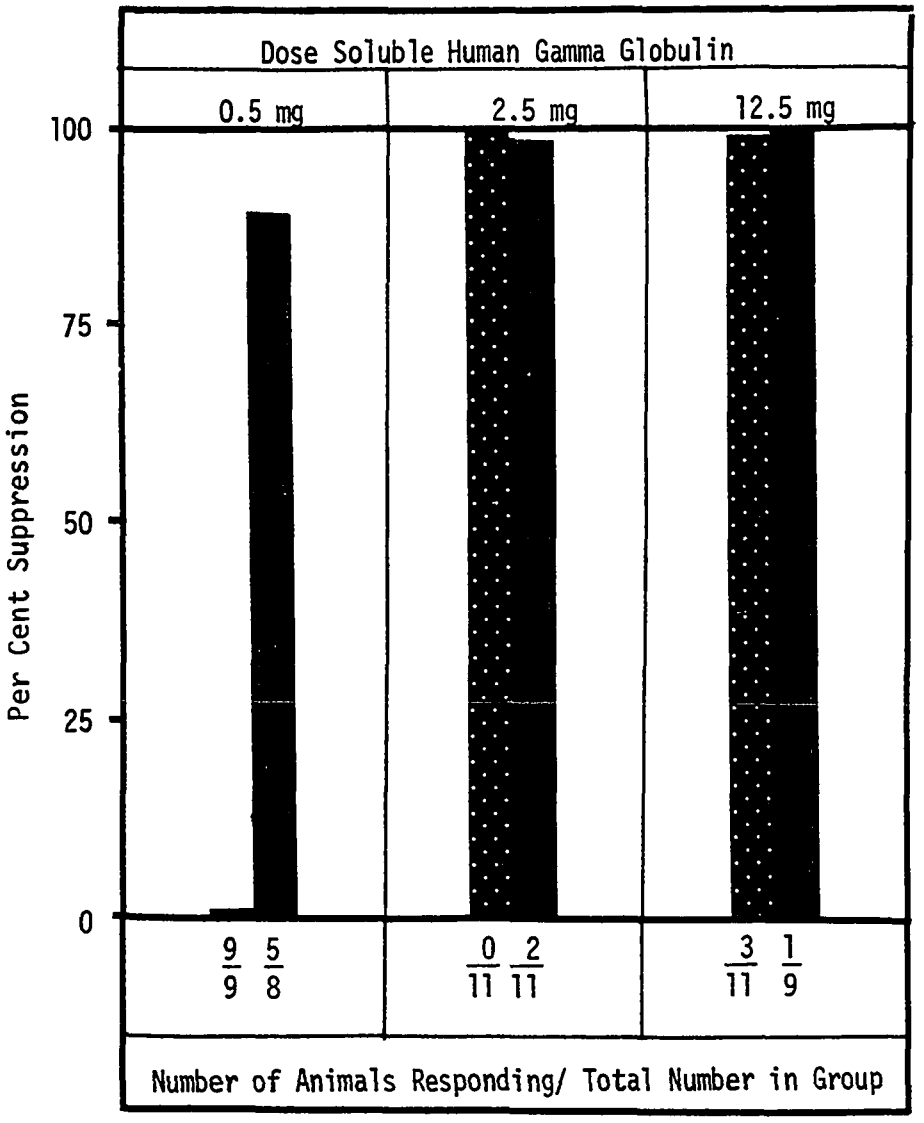

Figure 5. Per cent suppression of anti-human gamma globulin plaque forming cell response of male and female CBF_1 mice reconstituted with normal bone marrow cells and thymus cells taken from donors treated 10 days previously with soluble human gamma globulin. Number of animals reconstituted with normal bone marrow and thymus cells responding/ total number in group: female = 10/10, male = 22/24. Dotted bars - CBF_1 females, vertically striped bars - CBF_1 males.

influence on the susceptibility to the production of the unresponsive state.

Effect of Tolerant Thymus Cells on the Responsiveness of Normal Bone Marrow and Thymus Cells

The report by Gershon and Kondo (1971) that tolerant T-cells could suppress the anti-sRBC response of lethally irradiated mice reconstituted with normal thymus and bone marrow cells suggested that tolerant thymus cells produce a factor which is specifically immunosuppressive. If this were the case one would expect that C57BL/6 and CBF_1 thymus cells are more active than BALB/c thymocytes in producing such a factor. To test this hypothesis it was first necessary to determine if immunosuppression of the anti-HGG response of normal cells could be accomplished by simultaneous administration of HGG tolerant thymus cells. Mixtures of syngeneic tolerant and normal thymus cells were given to CBF_1 mice. Thymus cells taken from lethally irradiated donors were used in combination with normal thymus cells as a control for distribution of cells in the lymphoid tissue of the recipient. The following day the animals were injected with normal bone marrow cells and a primary challenge of heat aggregated human gamma globulin. Following a second aggHGG challenge, anti-HGG PFC responses were determined (Table 8). The mean PFC response of animals reconstituted with tolerant thymus cells in combination with normal thymus cells followed by normal bone marrow cells was lower than the PFC response of immunization controls (normal thymus cells plus x-irradiated thymus cells). It would appear, therefore, that unresponsive thymus cells can suppress the responsiveness of normal cells. However, due to the small number of animals in experimental groups the

TABLE 8

EFFECT OF TOLERANT THYMUS CELLS ON THE RESPONSIVENESS OF NORMAL BONE MARROW AND THYMUS CELLS

Cells Transferred[a]	Number of Recipients	PFC/10^6 Spleen cells[b]	Range
NT + XT + NBM	5	7.7	0.7 - 16.2
TT + NT + NBM	8	4.3	1.8 - 5.8
TT + NBM	11	0.6	0 - 3.4

[a]NT - normal thymus cells; XT - x-irradiated thymus cells; TT - tolerant thymus cells; NBM - normal bone marrow cells.

[b]Number of anti-human gamma globulin plaque forming cells per 1 x 10^6 nucleated spleen cells.

suppression observed may not be significant.

Effect of Tolerant Spleen Cells on the Responsiveness of Normal Bone Marrow and Thymus Cells

The number of cells in the thymus which are fully mature and capable of responding to antigenic challenge is relatively small. For this reason and because peripheralized immunocompetent cells may have different properties than those in the thymus, an experiment was performed to test the effect of tolerant spleen cells on the responsiveness of normal bone marrow and thymus cells. The experimental protocol was similar to that described for the previous experiment except that spleen cells were used as the source of tolerant or x-irradiated cells. Results are shown in Table 9. Animals grafted with tolerant spleen cells plus normal thymus and bone marrow cells had a mean PFC response (3.4 per 10^6 apleen cells) which was less than that detected in animals reconstituted with x-irradiated spleen cells plus normal thymus and bone marrow cells (4.8 per 10^6 spleen cells). However, this depression does not appear to be significant.

TABLE 9

EFFECT OF TOLERANT SPLEEN CELLS ON THE RESPONSIVENESS OF NORMAL BONE MARROW AND THYMUS CELLS

Cells Transferred[a]	Number of Recipients	PFC/10^6 Spleen Cells[b]	Range
NS + NT + NBM	10	30	9.6 - 120.0
TS + NBM	6	1	0 - 4.1
XS + NT + NBM	9	4.8	0 - 11.9
TS + NT + NBM	9	3.4	2.0 - 8.9

[a]NT - normal thymus cells; NS - normal spleen cells; TS - tolerant spleen cells; XS - X-irradiated spleen cells; NBM - normal bone marrow cells.

[b]Number of anti-human gamma globulin plaque forming cells per 1×10^6 nucleated spleen cells.

CHAPTER IV

DISCUSSION

This investigation was undertaken to determine if differences in the susceptibility to induction of tolerance in inbred mouse strains could be attributed to varying sensitivity to tolerance induction of thymus cells and/or bone marrow cells. Three mouse strains, BALB/c, C57BL/6, and the F_1 hybrid of a cross-mating of these two strains (CBF_1), were tested for unresponsiveness following injection of deaggregated human gamma globulin (sHGG). These mouse strains were chosen because it had been previously reported (Golub and Wiegle, 1969) that there was a marked difference in the susceptibility of BALB/c mice and C57BL/6 mice to induction of the unresponsive state with human gamma globulin, and that the F_1 hybrid of these two mouse strains had inherited the characteristic high sensitivity to induction of tolerance exhibited by its C57BL/6 parent. The results of the present investigation revealed that C57BL/6 and CBF_1 mice became tolerant with doses of sHGG as low as 0.5 mg. BALB/c mice were not tolerant unless they were treated with 12.5 mg tolerogen. These results agreed with those of Golub and Weigle that C57BL/6 mice were more sensitive to the production of the unresponsive state than were BALB/c mice and that the F_1 hybrid of these two mouse strains had inherited the sensitivity to tolerance induction of its C57BL/6 parent. Golub and Weigle were unable to induce

unresponsiveness in BALB/c mice at any tolerogen dose tested ranging as high as 10 mg. Discrepancies between their results and the observations reported here might be explained by variations in experimental protocol. Tolerogen preparations used in this investigation were centrifuged for 2.5 hrs at 100,000 X g, while Golub and Weigle prepared tolerogenic protein solutions by centrifugation at 20,000 X g for 30 min. Centrifugation at higher speed and for a longer period of time may have resulted in more efficient removal of aggregated material from the protein solutions. The Jerne plaque assay used in this investigation is a quantitative one which allows delineation of the degree of unresponsiveness induced in tolerogen treated animals. Golub and Weigle used the qualitative, immune elimination test to score animals as responsive or unresponsive. In the immune elimination procedure, an animal having a significant suppression of the PFC response might produce an amount of antibody sufficient to clear radiolabeled antigen and thus be scored as responsive. Because the BALB/c mice treated with 12.5 mg sHGG, although significantly suppressed, were found to be hyporesponsive, it is possible that by immune elimination they would have been considered as responsive.

Golub and Weigle suggested that differences in the susceptibility to tolerance induction of BALB/c and C57BL/6 mice might be attributed to the difference in the efficiency with which these mouse strains process trace amounts of aggregated material in tolerogen preparations for a subsequent immune response. This conclusion was based on the observation that their centrifuged tolerogen preparations contained aggregated material when it was subjected to analytical ultracentrifugation. They also noted that BALB/c mice could be rendered unresponsive

with lower doses of tolerogenic material which had been prepared by a sodium sulfate fractionation procedure. The sodium sulfate fractionated material did not contain aggregated material as judged by analytical ultracentrifugation. Hyde and Weigle (unpublished observations) found that phagocytes of BALB/c and C57BL/6 mice did not differ in their rates of engulfment of aggregated HGG nor did they differ in their ability to ingest antigen during tolerance induction. In addition, macrophages taken from tolerant animals were fully capable of participating in an immune response to the tolerated antigen. Neill, Cole and Hyde (1972) reported that C57BL/6 mice could clear intravenously injected carbon more efficiently than BALB/c mice. Because BALB/c and C57BL/6 mice apparently have phagocytes which are not functionally different, it was postulated that differences in susceptibility to tolerance induction of thymus cells and/or bone marrow cells may account for the differing sensitivities of these mouse strains to induction of the unresponsive state.

Experiments undertaken to determine the sensitivity of thymus cells and bone marrow cells to tolerance induction revealed that ten days after tolerogen administration C57BL/6 and CBF_1 thymus cells were significantly suppressed by the entire range of sHGG doses employed (0.5 mg to 12.5 mg). On the other hand, BALB/c thymus cells did not develop a high degree of tolerance until the animals were treated with 12.5 mg sHGG. The establishment of unresponsiveness in bone marrow cells was less easily achieved than was tolerance induction in thymocytes. After ten days exposure to tolerogen C57BL/6 and CBF_1 bone marrow cells had reached their maximum degree of suppression when treated with 2.5 mg

tolerogenic HGG. C57BL/6 bone marrow cells were 57.7 per cent unresponsive and CBF_1 bone marrow cells were suppressed 68 per cent by 2.5 mg sHGG. BALB/c bone marrow cells were 37 per cent tolerant when animals were treated with 0.5 mg sHGG, however at higher tolerogen doses unresponsiveness could not be detected in BALB/c bone marrow cells. Even though bone marrow cells of all three mouse strains were more refractory to tolerance induction than were thymus cells, it appeared that tolerance induction in bone marrow cells of CBF_1 and C57BL/6 mice followed a similar pattern; i.e., in both mouse strains maximum suppression of bone marrow cells occurred at comparable tolerogen doses. Results obtained by assaying bone marrow cell tolerance after 22 days exposure to tolerogen confirmed this observation. At this time period both CBF_1 bone marrow cells and C57BL/6 bone marrow cells were suppressed to the greatest extent at the 2.5 mg sHGG dose (63 and 85 per cent respectively). At the higher (12.5 mg) dose bone marrow from C57BL/6 and CBF_1 mice were only slightly less tolerant. It could be that differences in the degree of unresponsiveness observed between CBF_1 and C57BL/6 bone marrow cells reflect differences in the kinetics of tolerance induction in bone marrow cells in these two mouse strains. Chiller, Habicht and Weigle (1971) in studies of the kinetics of tolerance induction in bone marrow cells obtained 100 per cent suppression 21 days after tolerogen administration. However, complete tolerance was only observed at this one assay date. At other time periods between 8 and 42 days after injection of deaggregated HGG, bone marrow cells were found to be 60 to 80 per cent tolerant. Unresponsiveness in BALB/c bone marrow cells exposed to tolerogen for 22 days was inversely related to the dose of sHGG administered.

Nevertheless, even the maximum degree of unresponsiveness (67 per cent) observed in the bone marrow cells of BALB/c mice treated with 0.5 mg tolerogen did not exceed the suppression obtained in C57BL/6 and CBF_1 mice at higher doses. Suppression of BALB/c bone marrow cells by low but not high tolerogen doses might be accounted for by the fact that as the protein concentration of the tolerogen preparation increased the chance for contamination with aggregated gamma globulin increased as well. Kawaguchi (1970) reported that simultaneous injection of soluble bovine gamma globulin (sBGG) and aggregated bovine gamma globulin (aggBGG) interfered with the tolerogenic properties of sBGG. A concentration of aggBGG which was 1 per cent of the sBGG dose was effective in preventing the induction of the unresponsive state. Gershon and Kondo (1970) reported that some mouse bone marrow cells did not require T-cell helper activity in the anti-sRBC response. If this is the case in the anti-HGG response, one could postulate that such cells were primed by contaminating aggregated material in tolerogen preparations. Observations of tolerance induction in the bone marrow cell populations of these three mouse strains confirmed the observation of Chiller, Habicht and Weigle (1971) and Weigle, Chiller and Habicht (1972) that tolerance induction in these cells required a higher dose of tolerogenic material and a longer induction period than was necessary for the establishment of an unresponsive state in thymus cells.

Comparison of the levels of unresponsiveness attained in either thymus or bone marrow cells with those observed in intact animals at various sHGG doses revealed that, for all three mouse strains tested, whole body tolerance was reflected in the susceptibility of thymocytes

to the induction of tolerance. Similar results were reported by Chiller, Habicht, and Weigle (1971) who found tolerance in the A/J mouse strain to be a function of the sensitivity of thymus cells to the induction of the unresponsive state. The present investigation has shown, further that bone marrow cells from mouse strains which are highly susceptible to tolerance induction are likewise more sensitive to tolerogen treatment than are bone marrow cells from a mouse strain which is more refractory to induction of unresponsiveness.

The mechanism of the production of the tolerant state upon the interaction of antigen with immunocompetent cells is not known. Alternative views state that in the unresponsive animal there may be a destruction of cells capable of responding to the tolerated antigen, that clones of cells may be specifically paralysed but still be living, or that enhancing antibody blocks the immunologic potential of immunocompetent cell clones.

Studies of the kinetics of induction of and escape from tolerance might provide a clue to the mechanism of the unresponsive state. A rapid inactivation of cells implies that there has been a direct interaction of antigen with cell surface receptors resulting in paralysis or death of the cells. On the other hand, a prolonged period for tolerance induction would suggest that some active process is involved in the production of the unresponsive state. The observation of Chiller, Habicht and Weigle (1971) that there is a distinct difference in the kinetic pattern of induction of and escape from immune tolerance between T and B lymphocytes may mean that different mechanisms of induction are operable in each of these two cell populations. Rapid inactivation of

thymic lymphocytes within the first twenty four hrs after tolerogen administration suggests that these cells are killed or inactivated upon direct interaction of antigen with receptor sites on the T-cell membrane. Differences in the sensitivity of thymus-dependent cells of BALB/c, C57BL/6 and CBF_1 mice to tolerance induction might then be attributed to differences in the avidity or concentration of receptor sites on these cells. More efficient binding of tolerogen by C57BL/6 and CBF_1 thymocytes could result in a greater inactivation or elimination of clones of cells reactive to that antigen. Alternatively, these cells may require fewer hits with tolerogenic antigen for the subsequent production of the unresponsive state. Inactivation or death of thymus-dependent lymphocytes in tolerant animals may in itself explain why C57BL/6 and CBF_1 bone marrow cells became tolerant more easily than did BALB/c bone marrow cells. Because the end result of antigen exposure may reflect the outcome of competition between tolerogenic and immunogenic forms of the same antigen, any event which eliminates helper activity for immunization may increase the tolerogenic potential of the injected material. Due to the fact that CBF_1 and C57BL/6 thymus cells become tolerant at low antigen doses, one might expect a lower threshold for tolerance induction in the bone marrow cell population of these two mouse strains due to elimination of T-cell helper activity. It would be interesting to know if tolerance can be induced to HGG in bone marrow cells in neonatally thymectomized or irradiated, thymectomized, bone marrow repopulated mice, and if in such animals, strain variation such as that observed in this investigation occurs. The observation of Chiller, Habicht and Weigle that there is a delay in the production of the unresponsive

state in bone marrow cells of about one week following administration of deaggregated HGG suggests that some sort of active process must occur before tolerance can occur in this population of cells. Gershon and Kondo (1970) reported that bone marrow-derived lymphocytes which depend upon the interaction of antigen with T-cells before they are triggered for antibody production also require the presence of thymus cells to be rendered specifically unresponsive to the sRBC antigen. These workers (Gershon and Kondo, 1971) found that adoptive transfer of spleen cells from mice which were tolerant to sRBC would specifically prevent cooperation of normal thymocytes and normal bone marrow cells. They suggested three mechanisms whereby such an immunosuppressive effect by tolerant cells might be obtained: first, free antigen may be transferred along with the tolerant cells; second, the effect may be due to a product of tolerant bone marrow-derived cells; or third, the effect may be due to a product of tolerant thymus-derived cells. They ruled out the possibility that they were transferring antigen in their spleen cell preparations since spleen cells of thymus-deprived mice treated with tolerizing regimen of sRBC did not produce adoptive tolerance. Although it is known that antibodies can interfere with the immune response, Gershon and Kondo believed that passively administered antibody was not operable in their experiments. They obtained suppression when spleen cells of donor mice were not producing antibody and when there was no detectable serum antibody in donor animals. Spleen cells from animals which were producing antibody did not cause immunosuppression. Because high affinity antibodies are better able to interfere with antibody production, it seemed unlikely that the small amount of unmeasurable

antibody which may be present in the tolerant animal could be causing this effect in light of observations that partially tolerant animals produce antibody of low affinity. By a process of elimination of other mechanisms, these authors proposed that immunosuppression occurred due to a product of thymus-derived lymphocytes. They suggested that tolerant thymus cells produce an antigen-specific, immunosuppressive substance which they named IgY. The fact that several different investigators have been unable to abrogate the tolerant state by adoptive transfer of normal, syngeneic lymphocytes into tolerant animals lends support to this hypothesis (Crowle and Hu, 1969; Tong and Boose, 1970; McCullagh, 1970a; Chiller and Weigle, 1972). In addition McCullagh (1970a) reported that tolerance was induced in the transferred normal spleen cell population within three days residence in the tolerant host. Tong and Boose (1970) found that immunized spleen cells could not break tolerance in the unresponsive animal, thus it seemed unlikely that residual tolerogenic material could account for the unresponsiveness produced in the transferred cell population. Another observation favoring the hypothesis that residual tolerogen was not the factor which had rendered normal cells unresponsive was the report by McGregor, McCullagh and Gowans (1967) that normal cells will restore immunocompetence to x-irradiated, tolerant animals. In addition, Tong and Boose (1970) found that serum from tolerant mice caused a significant inhibition of the immune response of normal mice. However, spleen cells from these same tolerant donors did not inhibit the immune response after transfer to normal mice. If tolerant thymus cells produce an immunosuppressive factor, such as the IgY postulated by Gershon and Kondo, one would expect that thymus

cells of CBF_1 and C57BL/6 mice are more active producers of this substance or that they are triggered more easily to elaborate this material. Experiments employing transfer of mixtures of tolerant and normal cells provided suggestive evidence that tolerant thymus and, to a lesser degree, tolerant spleen cells could interfere with the responsiveness of normal thymus and bone marrow cells. It should be noted, however, that our experimental design was quite different from the elaborate double transfer system used by Gershon and Kondo (1971). For this reason direct comparison of their results with the results reported here may not be valid. The conclusion that T-cells do or do not produce an immunosuppressive substance must, therefore, await more exhaustive experimentation.

That the mechanism of tolerance induction in T-cells and B-cells may be different has been borne out in studies of tolerance to the thymus-independent antigen pneumococcal polysaccharide (SSS) (Howard, 1972). Immune tolerance induction with SSS was found by Siskind and Howard (1966) and by Matangkasombut and Seastone (1968) to be preceded by a phase of weak immunity. Such an immune phase has not been found during induction of tolerance in mice to thymus dependent antigen (Chiller and Weigle, 1971). Howard (1972) suggested that, even though this phenomenon might be attributed to the prolonged period required for tolerance in bone marrow cells, there were reasons to believe that the evanescent period of antibody production was related to subsequent induction of unresponsiveness to pneumococcal polysaccharide. The inverse relationship between molecular size of antigen and its tolerogenic properties which exists with thumus-dependent antigens does not hold true for pneumococcal polysaccharide. Howard, Zola and Courtenay (1971)

found that as SSS was broken down into small fragments by heating aqueus solutions of the material under pressure, it lost its immunogenic properties as well as its tolerogenic potential. Antigen which remains in SSS tolerant mice is fully able to induce immunity in normal mice (Howard and Siskind, 1969; Howard et al. 1970) in contrast to results which show that protein antigens in the blood of tolerant animals are tolerogenic (Frei et al. 1965; Golub and Weigle, 1969). It has been reported that animals primed with thymus-dependent antigens have an increased threshold to induction of the unresponsive state with the priming antigen (Cerny and Ivanyi, 1966). On the other hand, animals primed with SSS show minimal changes in their threshold to tolerance induction. Howard (1972) suggested that the time required to induce tolerance in bone marrow cells is excessive if one assumes that determinant-receptor interaction is the sole requirement, and that such a delayed onset of tolerance in this cell population suggests that antibody formation is a mandatory step for bone marrow cell tolerance. Studies of tolerance induction with SSS in strains of mice which vary in the development of an unresponsive state to this antigen might shed further light on the mechanism of tolerance induction in bone marrow-derived cells since this antigen eliminates confusion caused by concomitant tolerance induction in thymus-derived cells. Such studies may help elucidate any mechanistic differences which might occur during induction of tolerance with thymus-dependent and thymus-independent antigens.

In vitro studies by Feldmann and coworkers (Diener and Feldmann, 1972) on the induction of tolerance have helped determine the possible mechanism of tolerance in bone marrow-derived lymphocytes. These

investigators utilized the flagellar antigens of Salmonella adelaide which are available in three different forms: polymerized flagellin (POL, m.w. n x 40,000), monomeric flagellin (MON, m.w. 40,000) and a cyanogen bromide digest of flagellin, fragment A (m.w. 18,000). The polymerized form of this antigen was shown by Diener, O'Callaghan and Kraft (1971) to be thymus-independent, and for this reason, it allows investigation of tolerance at the B-cell level. As was found during in vivo studies with SSS, in vitro immunogenicity of these various flagellin preparations was directly related to their molecular weight (Diener and Feldmann, 1970) and in vitro, POL, the most immunogenic of these three antigens, was also the most tolerogenic. Preincubation of spleen cells with supraimmunogenic concentrations of POL followed by washing and 4 days incubation in the presence of immunogenic concentrations of POL resulted in unresponsiveness. Fragment A which was found to be a potent tolerogen in vivo by Ada and Parish (1968) failed to induce tolerance in vitro. Diener and Feldmann also failed to obtain low zone tolerance in vitro using these antigens, although they are known to produce such an effect in vivo. Thus, it was apparent that some mechanism which was operable in producing low zone tolerance and tolerance to fragment A was missing under in vitro conditions.

Further insight into the structural requirements for tolerogenic antigen in vitro came from the work of Feldmann (1972a). He observed that only DNP-POL could induce tolerance to the DNP hapten in vitro. DNP-HGG, DNP-BSA and DNP-MON were not tolerogenic. Feldmann noted, in addition, that tolerance to DNP only occurred when there was a large number of DNP residues conjugated to POL. The requirement for a high

hapten density suggested that induction of tolerance required formation of many closely spaced bonds with receptors on the surface of the immunocompetent cell.

In order to explain the potent tolerogenic activity of fragment A in vivo, Diener and Feldmann suggested that the additional mechanism provided by the intact animal may cause interlinking between monomeric antigen units. A highly likely candidate for such interlinking activity was specific antibody. These workers (Feldmann and Diener, 1970) reported that at a critical ratio of antigen to antibody in vitro suppression of antibody formation could be obtained with low antigen concentration. The immune suppression was due to a central effect and was entirely different from the immunosuppressive activity produced by excess antibody which prevents induction of tolerance while suppressing immune induction. Feldmann and Diener (1972) reported that only divalent antibody was effective in producing antibody-POL mediated immune suppression. In addition, fragment A, which was not tolerogenic by itself in vitro, when in combination with the appropriate concentration of specific antibody, produced unresponsiveness in vitro.

Successful antibody mediated tolerance induction in vitro with fragment A suggested the possibility that such a system may work with other antigens, such as BSA, where in vitro attempts at producing tolerance have been unsuccessful, whereas such antigens are efficient tolerogens in vivo. Diener and Feldmann (1972) were able to induce tolerance to ultrasonically fragmented sRBC provided that specific antibody was present at defined concentrations. Likewise, Diener and Feldmann (1972) have obtained antibody-mediated tolerance using chicken gamma globulin

as antigen. Since they could demonstrate antigen-antibody mediated tolerance with a variety of antigens, these workers concluded that it was a general immunologic phenomenon and was not restricted to Salmonella flagellar antigens. Two reasons were set forth by Diener and Feldmann to support the contention that in vitro, antibody-mediated, low zone tolerance is similar to that observed in vivo. First, concentrations of antigen necessary for the induction of in vivo and in vitro low zone tolerance are similar and second, the lag period required to elicit low zone tolerance is most easily explained by the necessity for antibody production prior to induction of the unresponsive state. These investigators have postulated that when the number of antigen-recognition sites cross-linked by antigen exceeds a critical threshold tolerance ensues. Cross-linking below this critical threshold level induces immunity. Diener and Feldmann believe that monomeric antigen complexed with antibody can serve as a lattice of repeating determinants in the same manner as polymeric forms of the same antigen.

If similar mechanisms apply to tolerance induction in bone marrow-derived cells by protein antigens such as HGG and thymus-independent antigens such as POL, several alternative hypothesis may be proposed to account for differences in susceptibility of bone marrow cells from different mouse strains to tolerance induction: 1) bone marrow cells may have antigen recognition sites of varying avidities; 2) the critical number of receptors which must be cross-linked to produce the tolerance "signal" may vary among strains of mice; 3) bone marrow cells from different mouse strains may have different membrane densities of antigen receptors; or 4) the amount of antibody produced by certain

strains of mice upon interaction with tolerogen may reach the critical concentration needed for antibody-mediated tolerance, while in other strains of mice there may be too much or too little antibody present for such immune suppression to occur. Further investigations of tolerance induction in BALB/c, C57BL/6 and CBF_1 mice may provide some clue as to the nature of the unresponsive state in bone marrow and bone marrow-derived cells. While the lag noted during the induction of bone marrow cell tolerance in mice suggests that antibody mediated suppression may occur in this cell population, it should be pointed out that peripheralized bone marrow-derived cells may exhibit a kinetic pattern of induction of the unresponsive state different from that of cells within the bone marrow. Chiller and Weigle (1972) presented data which suggested that peripheralized B-cells become tolerant within three days of injection of deaggregated human gamma globulin. Such results reveal the need for detailed investigations of tolerance induction in lymphocytes resident in both central and peripheral lymphoid tissue, as well as delineation of the effect of the presence or absence of one cell type during induction of tolerance in cells of the opposite type.

The fact that tolerance can be induced in CBF_1 mice as easily as in their C57BL/6 parent suggests that ease of tolerance induction is a dominant characteristic. These data may seem to fit a phenomenon which could be related to the inheritance of a single gene. However, Golub and Weigle (1969) in preliminary investigations of the F_2 and backcross mice of these same mouse strains ruled out the possibility that such inheritance was controlled by a single gene. Indeed, the differences observed in the induction of the unresponsive state in male

and female CBF_1 mice in this investigation suggests that the production of unresponsiveness may depend on the balance between many factors. Nevertheless, apparent dominance of the trait, whether it be due to one gene or several genes, implies that the mechanism of the induction of tolerance is a positive event.

CHAPTER V

SUMMARY

A comparative study of three mouse strains which vary in their susceptibility to induction of immune tolerance with human gamma globulin was undertaken to evaluate the cellular basis for the differing sensitivities of these strains to the induction of the unresponsive state. BALB/c mice became tolerant only when treated with 12.5 mg of a highly tolerogenic, ultracentrifuged preparation of human gamma globulin (sHGG). C57BL/6 and CBF_1 (BALB/c x C57BL/6) mice were significantly suppressed by doses of sHGG as low as 0.5 mg. Tolerance observed in intact animals of all three mouse strains was reflected in the sensitivity of thymus cells to the induction of unresponsiveness. Bone marrow cells from each of the mouse strains were more resistant to the production of tolerance than were thymus cells. Bone marrow cells assayed after 22 days of exposure to sHGG exhibited a higher degree of suppression than when they were assayed 10 days following administration of sHGG. Bone marrow cells of C57BL/6 and CBF_1 mice were suppressed to a greater extent than were similar cells taken from BALB/c mice. In addition, maximum suppression of C57BL/6 and CBF_1 bone marrow cells occurred at the same dose of tolerogen (2.5 mg). It was concluded that CBF_1 mice inherited the sensitivity to tolerance induction exhibited by thymus cells and by bone marrow cells of their C57BL/6 parent. When x-irradiated recipients were

grafted with tolerant thymus or tolerant spleen cells in combination with normal thymus and normal bone marrow cells, a slightly reduced number of plaque forming cells was observed as compared to controls.

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