STIMULATION OF CELL-MEDIATED IMMUNE RESPONSES IN MICE

SELECTIVE STIMULATION OF CELL-MEDIATED IMMUNE RESPONSES IN MICE IMMUNOSUPPRESSED BY A GRAFT-VERSUS-HOST REACTION

by

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ABSTRACT

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The immunosuppressive effect of the graft-versus-host (GVH) reaction was studied in F_1 mice injected with parental lymphoid cells. Suppression of the humoral immune response to sheep red blood cells (SRBC) in mice undergoing GVH reactions (GVH mice) was shown to depend upon the strength of the GVH reaction and its duration at the time of sensitization with SRBC. Adrenalectomy and castration of mice prior to the induction of a GVH reaction did not reduce the immunosuppressive effect, suggesting that immunosuppression results from immunological factors rather than from a general stress response.

Appropriate stimulation of GVH mice was capable of eliciting cell-mediated immune reactions to two different types of antigens while the humoral immune responses to the same antigens remained suppressed. Multiple challenges of GVH mice with allotransplantation antigen caused a rapid rejection of subsequent skin grafts from the same donor strain, but failed to stimulate the production of any detectable antibodies to

the transplantation antigens. Sensitization of GVH mice with SRBC in Freund's complete adjuvant stimulated a delayed hypersensitivity response to SRBC, although no humoral response to SRBC could be detected, even after three challenges with SRBC.

A single challenge of GVH mice with the thymus-independent antigen LPS did not elicit a humoral response, but a second and third challenge with LPS resulted in the production of a significant number of LPS-specific plaque forming cells and a high titre of anti-LPS hemagglutinating antibodies.

The experimental results are discussed in terms of a proposed model for the regulation of normal immune responses.

RESUME

STIMULATION SELECTIVE DE LA REPONSE IMMUNOLOGIQUE CELLULAIRE CHEZ DES SOURIS EN ETAT D'IMMUNOSUPPRESSION INDUITE PAR LA REACTION DU GREFFON CONTRE L'HOTE.

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L'effet immunosuppresseur de la réaction du greffon contre l'hôte (GVH) a été étudié chez des souris hybrides F_1 injectées avec des cellules lymphoides d'origine parentale. Il a été observé que le degré de suppression de la réponse humorale aux globules rouges de mouton (SRBC) causée par la reaction GVH varie suivant la vigueur de la réaction GVH et aussi en fonction de l'intervalle séparant l'induction de la GVH de la stimulation antigénique. L'adrenalectomie et la castration des souris hybrides F_1 avant l'injection des cellules parentales n'ont pas réduit l'intensité de l'immunosuppression suggérant que celle-ci est due à des facteurs immunologiques plutôt qu'à un etat de stress.

La stimulation de souris en réaction GVH (souris GVH), sous des conditions appropriées, avec deux types différents d'antigènes a provoqué une réponse immunologique cellulaire à ces antigènes alors que la réponse humorale est demeurée supprimée. Des injections répétées

d'antigène de transplantation a provoqué chez des souris GVH un rejet rapide d'une allogreffe de peau provenant de la même lignée de souris que les antigènes utilisés pour la sensibilisation. Toutefois il n'a pas été possible de mettre en evidence dans le serum de ces souris des anticorps dirigés contre les mêmes alloantigènes. L'injection à des souris GVH de SRBC melangés à de l'adjuvant complet de Freund a stimulé une réaction d'hypersensibilité retardée aux SRBC mais pas de réponse humorale ce même après trois injections de SRBC.

Une injection de LPS, antigène thymique-indépendant, à des souris GVH n'a pas stimulé la reponse humorale. Toutefois, deux ou trois injections de LPS ont induit la formation d'un grand nombre de cellules formant des plaques spécifiques au LPS et d'un fort titre d'anticorps hémagglutinants anti-LPS.

Sur la base de ces résultats un modèle sur la régulation de la réponse immunologique est proposé dans la discussion.

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PREFACE

The immunosuppressive effect of the graft-versus-host reaction is well documented and has been the subject of numerous studies performed in this laboratory. Although several studies had demonstrated prolonged survival of skin allografts on GVH animals, there was no evidence as to the effect of transplanting allogeneic stem cells into GVH animals. It seemed possible that allogeneic bone marrow might not be rejected when transplanted into GVH mice, and that the stem cells might develop into a clone of immunocompetent cells which could restore immunocompetence to the GVH host. Such an effect would also provide a means of inducing tolerance to specific histocompatibility antigens as well as studying the acquisition of tolerance to host tissue by the immunocompetent stem cells. To my surprise, the injection of allogeneic bone marrow into GVH mice stimulated cell-mediated reactions against the alloantigens rather than inducing tolerance to them. An attempt to stimulate a humoral response in GVH mice using similar treatment was unsuccessful. Although both these findings were unexpected, they seemed to provide a new approach for studying the immunosuppressive effect of the GVH reaction.

As the experiments progressed they illustrated several basic differences between cell-mediated and humoral immune responses which applied to normal mice as well as GVH mice. It became tempting to analyze these observations and speculate as to their causes in terms of what we presently know about the regulation of immune responses.

As a result the discussion ends with a proposed mechanism for the control of immune responses in both normal and GVH animals. Although this model is highly speculative, I feel that it provides a plausible explanation for immune regulation based on the observations from these experiments and our current understanding of immune responses.

CHAPTER 1: GENERAL REVIEW

Introduction

Although history records several early clinical applications in the field of immunology, the science itself is very new. As so frequently happens, these procedures were based on empirical observations and were employed with little or no understanding of how they worked. A classic example is that of Edward Jenner who in 1798 introduced vaccination against smallpox after observing that milk maids previously infected with nonvirulent cowpox never contracted smallpox (111). His technique of vaccination was widely used for a century before the virus causing smallpox was identified, and even longer before the mechanisms of immunity were understood.

The term "immunity", in fact, merely reflects a basic observation rather than an understanding of any of the principles involved. Strictly speaking, immunity may be defined as " a state of being exempt from penalty, duty, injury or harmful influence " (240). In a biological sense it refers to the immunity of living organisms to harmful agents, regardless of the nature of the agents. Immunology as a true science, however, goes far beyond this observation, and has its origins in the work of Koch who first identified and cultured the causative agents of infectious disease.

The early uncertainty and vagueness concerning immunologic phenomena are illustrated by many basic terms still in use today. The term "antigen" was originally defined as a substance which can stimulate an

animal to generate cells or proteins (antibodies) capable of reacting with it in a specific manner. The term "antibody" in turn refers to the substance or body formed to react against the antigen.

The science of immunology developed along two different lines, that of bacteriology and that of transplantation, which for many years seemed unrelated but then merged following the work of Loeb in 1930 and that of Medawar in 1945. By far, most of the early definitive work was done in the field of bacteriology. Significant breakthroughs came in 1890 when von Behring developed a specific diphtheria antitoxin and thereby discovered antibodies (16): and amazing intuitive insight was shown by Ehrlich in 1896 (62) when he proposed his theory of antibody formation which is surprisingly close to our present concept of antibody formation.

Although the study of transplantation immunity developed much more slowly, its importance became increasingly evident and it now forms the major aspect of immunological research. Since the graft-versus-host reaction is a type of transplantation reaction, the major emphasis of this review will be on transplantation immunity.

The earliest recorded suggestion of tissue transplants appears in ancient Egyptian and Greek writings (198) before the birth of Christ. These writings describe skin autografts taken from one location in order to repair mutilated parts such as lips and noses. Suggestions of whole organ transplants appear in many legends such as the classical legend of Cosmos and Damian* , two saints who were said to have removed the gangrenous leg from a faithful churchgoer and replaced it with a healthy leg from a dead Ethiopean.

*Golden Legend by Jacobus de Voragine, 13th century, also from a painting by Fra Angelico, Museo di San Marco, Florence.

The successful transplantation of whole organs did not occur until after 1902 when Carrel reported a technique for anastamosing large blood vessels to one another (38). Using this technique he performed successful autografts in dogs of the thyroid gland (39), kidney (40) and ovary (42,43). Surgical success was also achieved with transplants of these organs from one individual to another (allografts), although the eventual failure of these grafts was attributed to technical errors; and observations during the period of rejection were not published. Carrel also reported the first canine heart allograft that was implanted into the neck of the recipient and which beat normally for 2 hours (41).

The Genetic Basis of Transplantation.

A clear understanding of the genetic basis of tumor transplantation came into view in the early 1900's but these principles were not applied to whole organ transplants until some time later. In 1908, Bashford, Murray and Haaland (14) noted that a mouse tumor transplanted into a rat survived for an initial period of 8 or 9 days before being destroyed, but that a second mouse tumor implanted into the same rat survived only 3 or 4 days. From these results they postulated that the initial exposure to the mouse tumor had provided the rat with an immunity which allowed it to destroy a second transplant more rapidly.

Noticing a similarity between neoplastic and embryonic tissue, Rouss and Murphy (194,166) in 1911 found that rat sarcoma was tolerated and grew rapidly when injected into 16 day old chick embryos. Furthermore the injection of spleen tissue, and to a lesser extent bone marrow,

from adult chickens injected into the other side of the embryo receiving the tumor brought about a rapid retrogression of the tumor. In these experiments, Murphy not only introduced the idea of tolerance, but also identified adult tissue as the basis for the rejection of foreign neoplastic tissue. Carrying this one step further, Murphy (167) was able to suppress the rejection reaction and achieved increased survival of mouse sarcoma when injected into adult rats which had been previously treated with subcutaneous injections of benzene or whole body radiation.

Completely successful transfers of tumors among members of the same species, especially mice were not achieved until syngeneic strains were developed. A syngeneic strain can be defined as one that has undergone sufficient generations of brother x sister matings so that all the animals in any one strain (and of the same sex) are homozygous and genetically identical. Members of a given strain and sex will accept tumors, and indeed any tissue from members of the same strain and sex without the risk of immunological rejection.

Further insight into the genetic basis for tumor rejection was provided by Little (128) in 1914 when he proposed that tissue characteristics are determined by numerous genetic factors, and explained in a Mendelian fashion how phenotypic expression of these characteristics can be lost through successive matings. Using this theory, Little and Tyzzer (130) explained why a carcinoma which grew only in Japanese waltzing mice would also grow in animals of the F_1 generation of a

hybrid cross between these mice and those of another strain, but would not grow in animals of the F_2 or F_3 generations. They proposed that susceptibility to the transplantation of tumor is based upon a number of inherited factors and that possession of these factors, even in a half dose as in F_1 animals, renders the individual susceptible to tumor growth. Animals of subsequent generations, however, which lack one or more of these factors are not susceptible.

In 1922 the studies of Little and Johnson (129) showed that F_1 hybrids of two inbred strains readily accept tissue grafts from animals of the same sex from either parental strain. Subcutaneous spleen implants from waltzing mice into F_1 hybrids of waltzing and albino parents grew in the hybrids, while spleen implants from the hybrids did not persist in the waltzing mice. They postulated that the failure of the F_1 graft in the waltzing mice resulted from the expression on the F_1 graft of albino factors which were not present in the waltzing recipients. From this study and a similar study of his own, Bittner (24) concluded that retention of tissue implants is dependent upon the presence in the genetic makeup of the host of all factors found in the genetic constitution of the graft. This idea has become a basic rule of transplantation.

Based on experiments in 1918 with different combinations of interrelated rats, Loeb (132) postulated that "individual differentials" were present in chemical form on the surface of all cells and that they were inherited independently in a Mendelian form of blending. Loeb, however,

was unable to demonstrate an accelerated rejection or the production of antibodies following repeated allografts of the same tissue type (133). As a result, he did not accept the concept of an immune reaction as the cause of transplant rejection, and as late as 1945 he still believed that rejection was caused by a "disharmonious condition" between incompatible differentials of the donor and host tissue (134).

However, the immunological nature of skin graft rejection became apparent to Gibson and Medawar (80) in 1943 when they treated a young woman with extensive burns of the chest. Grafts of skin were made with "pinches" of skin from her own thighs as well as her brother's, and biopsies were taken at regular intervals. A second set of "pinches" was applied from her brother fifteen days after the first operation. It was noted that, while the autografts grew well, the first set of allografts began to degenerate by the 15th day and were completely destroyed by day 23. With the second set of allografts they observed an accelerated degeneration, beginning immediately and becoming well advanced by the eighth day. Although no evidence was found that the breakdown of foreign epithelium was brought about by lymphocytes, the "time relationships and accelerated rejection of second set allografts suggested that the destruction of the foreign epithelium was brought about by a mechanism of active immunization".

Conclusive proof of the immunological nature of graft rejection came from a series of classical experiments by Medawar (140,141) in 1945. Sets of pinch skin grafts (autografts and allografts) were

placed on rabbits, and biopsies of each were examined at regular intervals. While the autografts survived, there was degeneration of the allografts which was accompanied by a massive invasion of lymphocytes and monocytes. Application of a second set of allografts from the same donor was followed by an accelerated rejection which was the same whether or not these grafts were placed in the same operative field as the first set. These results showed the temporal relationship of rejection, the involvment of lymphoid cells, and the systemic nature which are required of an immune response. The most important test, that of specificity, was shown when a set of allografts from a related donor rabbit was applied following the rejection of the initial allograft. Prior sensitization caused an accelerated rejection of the second set allografts, but did not accelerate rejection of the third party skin. Thus graft rejection demonstrated two essential features characteristic of an immunological reaction, namely immunological memory and specificity. It was therefore concluded that allograft rejection was mediated by an immunological phenomenon.

Histocompatibility Systems.

Although a Mendelian interpretation of graft survival was put forth by Little in 1914, the genes controlling these characteristics were not identified until 25 years later. Working with sarcoma in a pure line of albino mice, Gorer (86,87) identified isoantigenic factors responsible for the rejection of incompatible tumors and demonstrated specific iso-

agglutinins in the sera of mice in which tumors had recently regressed. He postulated that two dominant genes governed the transplantability of tumors in these strain combinations, and that the genes determining the presence of these isoantigenic factors might be identical with those determining antigenic differences in blood groups. It is interesting that Gorer's experiments also suggested that "there is some evidence that malignant cells are antigenically different from the tissues from which they arose, but such differences do not afford an efficient stimulus (for rejection) under ordinary conditions of transplantation".

Early studies of transplantation genetics were carried out in mice because of the homozygous inbred strains that were available By backcrossing F_1 hybrids of 2 purebred strains, Snell (220) in 1948 produced what were later called "congenic resistant" (CR) strains of mice. Mice of these strains were identical with established inbred stocks except for the presence of one recessive allele at a single locus. Using CR strains of mice Snell determined that at least 7 loci and probably 14 or more were involved in determining the fate of tumor transplants, and proposed the names "histocompatibility genes" (H genes) for the loci and "histocompatibility antigens" (H antigens) for their products. The prefix "histo" was used because he believed that the same genes determined susceptibility to or resistance to tissue transplants in general. After producing more of his congenic resistant strains, Snell (222,223) was able to distinguish between alleles of a single weak locus without stronger H systems interfering. Based on his tumor studies, Snell delineated individual H-loci in the mouse and assigned them the

symbols H-1, H-2 etc..

As it became apparent that neoplastic tissue can grow even in the presence of an immune response, normal tissue was used for histocompatibility studies and revealed antigenic factors which had not been previously revealed in tumor studies. In 1957 Barnes and Krohn (13) studied the survival of parental strain skin grafts on F_2 hosts and estimated that at least 15 H loci were responsible for skin graft rejection. More recently, H antigens have been detected using such tests as cytotoxicity, hemagglutination, complement fixation, and indirect fluorescent antibody tests.

By 1966, a minimum of 29 H loci were known to exist (10) widely distributed throughout the chromosomes (228), but that number may be a gross underestimate. Bailey (9) points out the complexity of these loci by suggesting that most are really nests of tightly linked loci and highly susceptible to mutation He estimates that the total number of H loci may be greater than 400.

The strongest and most extensively studied H-locus in the mouse was designated H-2 by Gorer et al. (89) who located it in the ninth linkage group close to a series of loci that cause fusing, kinking, and shortening of tail structure. Early estimates of 5 H-2 alleles (221) were revised upwards to 9 alleles (226) and at present more than 20 alleles have been described at the H-2 locus (228). The complexity of this region is further established by the fact that it is divisible by crossing over into at least 5 separate regions, permitting phenotypic expression of many antigenic specificities for each allele (225).

At present, 39 H-2 specificities have been serologically defined in the mouse and the number is likely to increase even more (58,225). Some of these antigens are shared by many different H-2 alleles and are present in most pure bred strains as well as wild mice. These are termed "public" specificities (116). Other antigens of the H-2 system are limited to one or very few alleles and are therefore present in very few strains of mice. These are termed "private" specificities.

It is now believed that the segment of chromosome which determines H-2 antigenic specificities is actually composed of a complex number of genes, some with functions not related to the H-2 antigens (117,118). This region, therefore, has been referred to by Bach (8) as the major histocompatibility complex (MHC). At either end of the MHC lie the two major serologically defined (SD) loci, H-2K and H-2D, with a recombination frequency between them of 0.4% (205,224). Between these SD loci lies the Ss-Slp locus identified by Shreffler (204) as being responsible for the production of two serum proteins.

Within the MHC, several lymphocyte defined (LD) loci have also been identified based on a number of lymphocyte activities which they control. To the left of the Ss-Slp and next to the H-2K locus lies the Ir locus which determines the ability of the animal to mount an immune response to a number of synthetic polypeptides (17,139). The Ir region seems to be a complex region containing a cluster of genes which are important in the immune response to many different types of antigens. In addition to controlling the response to synthetic polypeptides Ir genes have also been shown to control responsiveness to a

variety of allogenic antigens and several strong native antigens such as bovine serum albumin.

Several mutant strains of mice have recently been described as differing from the original strain by a spontaneous mutation in the MHC to the left of Ss-Slp (189,243). Although it is not possible to obtain cytotoxic or hemagglutinating antibodies against the difference by cross-immunization, there may be reciprocal and rapid rejection of skin grafts exchanged between the two strains (11). Despite the possession of identical SD antigens, the MHC difference was found to activate T lymphocytes *in vitro* as well as *in vivo*. The LD difference in the mutant strain was sufficient to cause reciprocal stimulation in mixed leucocyte culture (MLC) and cytotoxicity as measured by cell-mediated lympholysis. The *in vivo* counterpart of MLC reactions, namely the graft-versus-host (GVH) reaction has been demonstrated by Livnat and Klein (131) and by Rodney et al. (189) in several mutant strains of mice. They have shown that GVH reactions can occur in combinations which are serologically indistinguishable and which accept each other's skin grafts permanently. They attribute the GVH reactivity to a cross-over within the Ir region and speculate that the genes leading to the GVH and MLC reactions are identical with the Ir genes A somewhat complex relationship appears to exist between SD and LD regions in regard to their involvement in MLC and GVH reactions. It has been proposed that lymphocyte defined antigens stimulate MLC and GVH reactions while serologically defined antigens appear to be the targets of immunocompetent cells in these reactions (3).

The relative importance of the regions of the H-2 gene complex in eliciting GVH reactions has been summarized by Oppltova and Demant (173). GVH reactions are stimulated most efficiently when there is Ir-region incompatibility, but only weakly by H-2K, H-2D, and Ss-Slp incompatibility. These requirements closely resemble those for the MLC reaction, but seem to be quite different from the genetic requirements for skin graft rejection which can be caused by H-2K and H-2D incompatibility but not by Ir or Ss-Slp incompatibility.

It should be pointed out, however, that control of MLC and GVH reactivity in the mouse is not limited to the H-2 region. Festenstein, et al (69) have demonstrated a single genetic region known as the M locus which is not linked to the major histocompatibility system nor to several known H antigens. The antigens of the M locus cannot be detected serologically but can stimulate strong MLC and GVH reactions as well as allograft rejection (69,108,109).

Serologically defined H genes express themselves by producing H antigens on the surface of cell membranes. Also known as transplantation or alloantigens, the H antigens are glycoprotein in nature with the protein moiety accounting for 80-90% (203) and containing most, if not all of their antigenic determinants (186). LD genes are also believed to be phenotypically expressed at the cell surface although their phenotypic product is probably not of the same chemical nature as H antigens (8). Nonetheless, David and Shreffler (53) have produced several anti H-2K reagents which they have shown to contain antibodies directed against LD antigens. Using these antisera they

have tentatively identified 6 LD specificities determined by the Ir locus.

H-2 antigens have been demonstrated on virtually all types of tissues including skin, lymphoid tissue, myeloid tissue and neoplasms derived from these cell types. The primary function of transplantation antigens is not known, although many theories have been proposed. Jerne (112) cites the importance of H-antigens in ontogeny, namely in the cell to cell recognition that is needed for the formation of specialized tissues and for morphogenesis. Other researchers (98,161) suggest that H antigens function in cell contact and recognition phenomena, providing a surveillance mechanism capable of eliminating cell variants such as neoplasms which have a changed surface structure. Burnet (31,32) points out that susceptibility to malignant disease increases with the mother-fetus relationship of higher vertebrates, and suggests that H-antigens provide a means of recognizing and removing neoplastic cells which might be received from the mother. In regard to LD genes, Bach (8) has postulated that recognition of LD differences may be a key event which will eventually lead to the allograft reaction.

Histocompatibility systems have been found in a wide range of vertebrates including fishes (113), amphibia (101), birds (202) and many species of mammals (A good review of these is provided by Batchelor (15)). Although no attempts have been made to identify H systems among invertebrates it seems quite likely that similar "self" antigens also exist on the cells of lower animals. Such surface anti-

gens may play an important role in the morphologic development of all animals as well as the natural immunity which has been described in lower animal forms (110).

The Immune System

The Thymus Dependent System.

The most important cell in the immune response is the lymphocyte. Once thought to be a simple, homogenous population of cells, lymphocytes are now known to vary greatly with respect to origin, structure and function. Most evidence suggests that lymphocytes, and indeed all blood cells, develop from a single type of hemopoietic stem cell (144,162,164). These stem cells are first formed in the early yolk sac, probably from cells that migrate out of the anterior quarter of the primitive streak (165). Most stem cells in the yolk sac differentiate into early stage erythrocytes, but some divide to form new stem cells which are released into the general circulation. Early migration of the new stem cells to the liver results in the formation of "colonies" and the initiation of hepatic hemopoiesis which is evident in the mouse by the tenth day of fetal development (162). Although the earliest detection of stem cell colonization appears in the fetal liver, migration to the bone marrow and lymphoid organs is almost simultaneous. In the mouse, Moore and Owen (163) have demonstrated the presence of stem cells within the epithelium of thymic rudiments of 11 day embryos, while the earliest colonization of the bone marrow has been shown at 17 days gestation (143).

Stem cells from the yolk sac have been shown to be pluripotent, i.e. having a wide range of developmental capabilities (162). The particular cell type into which they differentiate is determined by the inductive environment of the organ rudiment in which they proliferate.

Two pathways have been identified for the development of lymphoid cells. One cell population migrates through the thymus (thymus-dependent system) and gives rise to what are termed thymus derived cells or T cells. In birds a second population of lymphoid cells (thymus-independent system) migrates through the bursa of Fabricius. In mammals, the route by which this second population of cells migrates has not been established and the thymic-independent cells are merely called bursal equivalent cells, or simply B cells. Stem cell migration originates directly from the yolk sac in early embryonic development, but once the bone marrow has developed, the marrow becomes the origin of migration and continues as such throughout the animal's life.

It is generally believed that cells within the thymus do not participate directly in immune reactions. As a primary lymphoid organ, however, the thymus is vitally important in the development and maturation of the immunocompetent cells which are necessary for these reactions. Evidence for this belief is shown by the fact that neonatal thymectomy in mice, rabbits and chickens renders them unable to mount an immune response in later life (85,146,238). In some cases, immunocompetence can be restored by grafting a syngeneic thymus into such animals (145).

Within the thymic environment, migrant cells proliferate and differentiate into thymus lymphocytes (thymocytes). In the mouse the

great majority of these thymocytes appear to die in situ within 3-4 days and fewer than 0.5% are released to the circulation (137,142). This excessive lymphopoiesis within the thymus may represent a reserve function of the thymus, or it may be that it provides an excessive number of lymphocyte mitoses needed to generate, on a random basis, lymphocytes with a wide range of antigenic reactivity patterns Those cells which are released from the thymus migrate to peripheral lymphoid tissue and are termed "thymus derived" lymphocytes or "T" cells. During their development in the thymus, mouse T cells acquire a distinct "theta" surface antigen which is unique to T cells and is frequently used as a surface marker to trace cell development. Recent studies have shown a similar human thymic lymphocyte antigen (HTLA) on human thymic derived lymphocytes (1,219). A comprehensive review of surface antigenic markers for distinguishing T and B cells in mice has been made by Raff (180).

The production of T cells and the development of immunocompetence is believed to be controlled by a humoral factor produced by the thymus. This was first shown by Osoba and Miller (175,176) who restored immunocompetence in neonatally thymectomized mice by the intraperitoneal implantation of thymus grafts enclosed in millipore chambers. Subsequently, extracts from calf thymic tissue were also found to restore immunocompetence and lymphocytopoiesis in neonatally thymectomized mice (242). Although the precise cause of this restoration has not been established, an active factor has been isolated from the thymic extracts. This factor has been termed thymosin by White and Goldstein (82) who

have evidence suggesting that it is produced by thymic epithelial cells. The injection of extract containing thymosin into normal newborn mice has been found to accelerate the development of T cell functions such as cell-mediated immune reactions and rosette formation (84).

The activity of thymosin does not appear to be limited to the thymus. Although most T cell maturation is initiated in the thymus, cells having the properties of T lymphocytes have also been shown to be produced in the peripheral lymphoid organs (82,83). Komura and Boyse (120,121) have demonstrated the ability of thymosin to specifically induce mouse precursor cells to differentiate into T lymphocytes in vitro Mouse bone marrow stem cells incubated for 90 minutes with a purified thymic extract were shown to acquire the characteristic T lymphocyte surface antigens specific for mature T cells. More recent reports (199,200), however, have indicated that such T cell differentiation can be initiated by agents that are not specific products of the thymus. Cyclic AMP and poly A:U have been found to induce in vitro the expression of T cell specific antigens on precursor cells found in the spleen and bone marrow of "nude" (congenitally athymic) and normal mice. In addition there has been some speculation that endotoxin (LPS) may be capable of promoting T cell maturation in vitro (200).

Although the majority of thymocytes appear to die without ever leaving the thymus, a significant number of T cells are released and "seeded" to the peripheral lymphoid organs. T cells appear to

leave the thymus by way of its lymphatic drainage system as well as through the rich supply of veins in the cortico-medullary junction (143) A small proportion of these mature T cells localizes in "thymic-dependent" areas of the spleen and lymph nodes (177). These areas are found within the lymphoid follicles of the spleen immediately surrounding the central arterioles in the mid and deep cortical zones of the lymph nodes. Studies by Raff and Owen (183) using anti-theta serum indicate that T cells account for 65-70% of the lymphoid cells in the lymph nodes and 30-35% of those in the spleen.

The largest percentage of existing T cells becomes part of the recirculating pool of small lymphocytes. T cells have been shown to constitute up to 85% of this pool, though only about two-thirds of these bear the theta antigen (152). In the adult, the recirculating small lymphocytes are carried in the blood to the thymic-dependent areas of the spleen and lymph nodes where they leave the blood through post-capillary venules. They circulate through the lymphatic system and return to the blood by way of the thoracic duct which, as a result, is an abundant source of T cells (91). The majority of recirculating lymphocytes have a long life span measured in the order of months in rats (188) and up to 10 years in man (30).

T cells play a primary role in cell-mediated immune reactions such as graft rejection and delayed hypersensitivity (143) The cells which participate in these reactions are specifically sensitized, and therefore capable of antigenic recognition. Considerable dispute has arisen over the nature of the recognition site on the sensitized T cell.

Whereas immunoglobulin receptors have been readily identified on the surface of B cells, similar techniques have failed to demonstrate their presence on T cells. Uptake of antigen by thymocytes *in vitro* is minimal compared to that seen with peripheral lymphocytes (34), and immunoglobulins have not been found on thymocytes by immunofluorescence or immunoautoradiography (184,236). Some indirect evidence, however, suggests that "immunoglobulin-like" receptors might be present on T cells. Antisera to immunoglobulins were found by Mason and Warner (136) to interfere with transplantation immunity and delayed hypersensitivity, functions usually performed by T cells. In addition, Greaves and Möller (93,94) have shown that sensitized thymus derived lymphocytes (possessing the theta antigen) bind to sheep red blood cells and form rosettes. Furthermore this binding can be inhibited by the prior treatment of these T cells with antisera directed against different parts of the immunoglobulin molecule (190,191). Greaves and Hogg (92) have suggested that the surfaces of T cells may contain immunoglobulin receptors which are only partially exposed, and are therefore difficult to detect by conventional means.

If T cells do possess immunoglobulins on their surfaces as recent evidence suggests, it is important to determine whether these immunoglobulins are actively produced by the T cells or passively acquired as cytophilic antibody. Recent reports by Roelants et al. (191,192) present evidence that T cells have immunoglobulin receptors for antigen and that they are actively synthesized by the cells carrying them. Both T and B cells from sensitized mice were found to specifically bind labeled antigen and "cap" with anti-immunoglobulin serum in cul-

ture. This was followed by endocytosis of the complexes and the disappearance of detectable receptors. After 18 more hours in culture the same T cells appeared to have resynthesized receptors, and once again bound specific labeled antigen and "capped" with anti-immunoglobulin serum. Recognition by the T cell of antigenic determinants results in the transformation of specific lymphocytes into large pyroninophilic blast cells which proliferate to produce a clone of small lymphocytes. Virgin antigen sensitive cells cannot take part in immune reactions, but must first be sensitized or "educated". By interacting with a specific antigen sensitive cell, the antigen thus stimulates the proliferation of a whole clone of cells capable of reacting with it. The process of education might be merely a quantitative expansion of the cells capable of reacting with a specific antigen, or it may bring about further differentiation of the T cell and the development of antigen-specific receptors with higher affinity.

Once educated, thymic derived lymphocytes can participate in cell-mediated immune reactions such as graft rejection. Until recently it was believed that sensitized T cells can directly act as killer cells in such reactions without the collaboration of bone marrow derived cells (148,231) New evidence, however, suggests that in addition to sensitized T cells, non-specific cells of bone marrow origin may act as effector cells in cell-mediated reactions (103,122,123).

T cells are also required to serve a "helper function" in reactions leading to the synthesis of antibodies against "thymic-dependent" antigens. By far, the majority of antigens fall into this category.

The actual antibody forming cell has been shown to be of bone marrow origin, but nonetheless dependent on T cell cooperation (149,154). The function of the T cell in such reactions may be to combine with the antigen and present its antigenic determinants to the B cell in a more stimulatory fashion (147). The addition of supernatant from cultures of nonspecifically activated T cells has been shown to activate B cells to produce specific antibody when they are cultured alone with a given antigen (4). This would suggest that the T cell may release a soluble factor which nonspecifically stimulates the B cell to produce antibody once it has bound with the antigen. Although Vitetta et al. (235) have demonstrated cells in the thymus which are capable of secreting immunoglobulin, these cells do not bear any thymic antigens and are believed to be plasma cells or bone marrow derived lymphocytes which comprise less than 2% of the thymic cell population.

Finally, T cells may also provide the means for immunological "memory" (50), a basic characteristic of immune responses. "Memory" seems to result from antigen induced proliferation of specific T cells and the consequent production of a T cell clone of long lived antigen reactive cells (158,193).

The Thymus-Independent System.

In 1621, Hieronymus Fabricius reported the existence of a previously undescribed organ of the chicken located at the caudal end of the cloaca (239). Experiments in the 19th. century showed that this

organ was present in at least 30 different species of birds. Furthermore, its development was found to roughly parallel the development of the thymus. Like the thymus the bursa develops from endodermal-ectodermal interaction, growing most rapidly soon after hatching and continuing its growth until sexual maturity, at which time it involutes (56,187).

As early as 1885, the bursa was identified as a center of lymphocyte activity (143). The first indication that the bursa was connected with immune reactions came from a study by Glick et al. (81) in which they determined that chicks which were bursectomized at hatching were found to be very susceptible to infection by Salmonella typhimurium and did not produce any detectable antibody.

While lymphoid colonization in the chick thymus starts at about 8 days incubation, colonization of the bursa does not start until day 13 or 14. Large, undifferentiated cells migrate from the yolk sac and spleen and localize within epithelial buds of the bursa where they become committed to the lymphoid line (143) The subsequent proliferation and maturation of these cells results in the establishment of a population of bursal small lymphocytes. The bursa of Fabricius has been identified as the first site of antibody formation. Thorbecke, et al. (232) have demonstrated IgM production in the chick bursa as early as the 18th. day of incubation, although this is not believed to arise as a result of antigen stimulation within the bursa.

Studies performed in the chick at the time of hatching have shown a migration of cells from the bursa to other lymphoid organs including the spleen and even the thymus (99). This migration continues up to

at least 6 weeks of age but ceases by the onset of bursal involution. Bursectomy at this time has very little effect on antibody production (237). Although a hormonal mechanism for bursal control of lymphopoiesis has been proposed (197), it has not been firmly established.

It has been more difficult, in mammals to identify a specific organ equivalent to the avian bursa of Fabricius. Recent studies (47,97) suggest that the Peyer's patches and other gut associated lymphoepithelial tissues may be the mammalian homologue of the bursa of Fabricius. There are several lines of evidence to support this: 1) removal of Peyer's patches in rabbits has been shown to result in a deficiency of antibody production; 2) Peyer's patches have lymphoepithelial relationships similar to those in the bursa; 3) unlike follicles of the spleen and lymph nodes, Peyer's patch follicles are not dependent upon antigenic stimulation for development, 4) Peyer's patches are well developed in animals that have been subjected to neonatal thymectomy; and 5) Peyer's patches have been shown to be composed mainly or entirely of thymus-independent lymphoid tissue.

Regardless of whether the Peyer's patches represent the mammalian equivalent of the bursa, a distinct population of marrow-derived, thymic-independent lymphocytes has been identified in mammals (154,169). These peripheral lymphocytes are termed bursal-equivalent or "B" cells.

B cells recirculate to a much more limited extent than T cells and with a much slower transit time (181). The great majority of B cells tend to settle in definite thymic-independent areas of the peripheral lymphoid organs. Some evidence suggests that B cells have

specific surface antigens which can be used to distinguish them from thymus derived cells (182). These "mouse-specific B lymphocyte-antigens" (MBLA) are believed to be somewhat similar to the thymus specific antigens although their existence and specificity are not nearly so well documented. Theta antigens are readily identified by specific antisera prepared in allogeneic mice. MBLA, however, have only been identified using antisera prepared in rabbits against lymph node cells from mice which have been thymectomized, lethally irradiated, and reconstituted with syngeneic fetal liver cells. Since the antiserum is directed against a xenogenic antigen(s) it is not certain whether it is solely directed against MBLA or what percentage of B cells express these antigens

When B cells are activated by antigen they divide and differentiate to produce expanded clones of antibody forming cells which usually form antibody of a single specificity (170,171). It is now believed that T cells play a major role as "helper" cells in such reactions with most antigens which are therefore termed thymic-dependent antigens. The first evidence for such T-B cell cooperation was supplied by Claman et al. (44) who found that irradiated mice given both thymus and bone marrow cells made a far greater antibody response to sheep erythrocytes than recipients of either thymocytes or bone marrow cells alone In such reactions, the T and B cells have been shown to bind to different determinants of the same antigen (156). As previously mentioned, cooperation is believed to be accomplished either by a bridging effect of the antigen between the T and B cell, or by the release by the T cell of a chemical mediator which enhances B cell responses (181). Some evi-

dence suggests that macrophages or adherent cells may also play an important role in this cooperation, either by processing and concentrating the antigen or by producing soluble factors which may be necessary for normal cell proliferation. A good review of some recent concepts in the control of antibody synthesis is presented by Bretscher (29).

Although the property of immunological memory is usually attributed to thymus derived cells, some memory function has been shown to reside in a population of long lived B cells (230). Expression of memory by B cells, however, may be dependent upon the presence of unprimed T cells (153).

A small number of antigens are known to be able to stimulate antibody production in B cells without the cooperation of T cells. Termed thymic-independent antigens, these include tetanus toxoid flagella H antigens of Salmonella adelaide and polysaccharides from E. coli and pneumococcus (150,157). Animals which have been neonatally thymectomized (100), lethally irradiated and reconstituted with bone marrow (6), or treated with anti-theta serum (60) show almost normal primary antibody responses when subsequently exposed to these antigens. Studies by Feldman and Basten (68) have suggested that thymic dependence or independence is related to the mode of presentation of the antigenic determinants rather than to their specificity. Thymic-independent antigens appear to have repeating determinants which can be presented to B cells and stimulate them directly. This in turn might suggest that the role of T cells as helpers in antibody production lies in their ability to present to the B cell sufficient antigenic determinants to

cause stimulation of antibody production.

Many thymic-independent antigens, when present in high concentrations, have been shown to activate B cells nonspecifically into increased DNA synthesis and polyclonal antibody synthesis (4). LPS, for example, in appropriate high concentrations is believed to exert this mitogenic effect on all B cells irrespective of the specificities of their antibody receptors (48). Coutinho and Möller (49) have explained the difference between specific B cell activation by thymus-independent antigens and their nonspecific mitogenic effect on the basis of a quantitative concept. Optimal (higher) concentrations of a mitogen presumably bind nonspecifically to B cell receptors which are not immunoglobulins, and they therefore activate a whole population of B cells, irrespective of the specificity of their immunoglobulin receptors. As the concentration of mitogen is decreased the number of mitogen molecules nonspecifically bound to the cell surfaces decreases below the threshold required for triggering. This results in a decreased proportion of responding cells until non-specific activation cannot be detected. At lower mitogen concentrations, all B cells will nonspecifically bind mitogen molecules at the same subthreshold level. In addition, however, B cells having specific immunoglobulin receptors for the antigenic determinants of the mitogen will bind a larger number of mitogen molecules and will become activated by the confrontation with the mitogenic properties carried by these molecules. At these low mitogen concentrations the responding cells are selected by their

specific Ig receptors, and the response is detected as a specific, thymic-independent immune response.

The ability of thymic-independent antigens to stimulate B cells directly circumvents the normal requirement for T and B cell cooperation. In the case of thymic-dependent antigens, stimulation of antibody production by the B cell depends upon the receipt of 2 signals by precursor cells (29). One signal is provided by the binding of antigen to specific receptors on the B cell of a thymic factor produced as a result of T cell interaction with the antigen. Möller et al. (160,217), have shown that T-B cell cooperation is not an obligatory event in the triggering of B cells and that LPS can be substituted for the T cell activity which is normally necessary for a response against SRBC. They showed that the primary PFC response to SRBC was very low in mice which had been thymectomized, lethally irradiated, and repopulated with syngeneic bone marrow. The injection of SRBC coated with LPS restored the PFC response to SRBC to control values, but the injection of SRBC and LPS separately had no effect. Thus, heterologous red blood cells were in effect converted into thymus-independent antigens by coating them with LPS.

The Graft-Versus-Host Reaction

Introduction.

For the first half of this century the primary emphasis of most transplantation studies was placed on the reaction of the host against

the grafted tissue; even when the grafted tissue consisted of immunologically competent cells. In 1916, Murphy (168) inoculated the chorioallantoic membranes of 7 day chick embryos with fragments of certain tissues including spleen, liver and bone marrow from adult chicken donors. He noted a considerable enlargement of the host's spleen and the appearance of white nodules on the membranes in the spleen and elsewhere, but attributed this to a reaction of the host spleen and leukocytic elements against the donor tissue. Subsequent studies (51,52) seemed to confirm this theory, and for 40 years such splenomegaly was believed to result from a unique ability of spleen, liver or bone marrow grafts to release sub-cellular protein constituents and stimulate a host-versus-graft reaction in the host spleen (61).

The first suggestion that grafted tissue could react immunologically against the host came in 1953 from independent studies by Dempster (59) and Simonsen (206,211). While studying the rejection of kidney transplants in dogs, both men noted the appearance of pyroninophilic cells infiltrating the renal cortex after several days' residence in the foreign host. Both men postulated that these cells were of graft origin and that they were involved in an immunologic reaction on the part of the graft against foreign host antigens. Although there is presently some dispute over the origin of these cells, the significance of these reports lies in their presentation of the concept of a graft reacting against the host. Such reactions are now termed "graft-versus-host" or simply GVH reactions, and are the

subject of a number of recent review articles (18,67,138).

The reactivity of immunocompetent grafts was illustrated in 1954 by Mitchison (155). He showed that the breakdown of lymphosarcoma in a non-susceptible strain of mice could be greatly accelerated by injecting the host with a lymph node suspension from syngeneic, immunized animals. The power to transfer immunity was confined to the lymph nodes draining the site of implantation. In 1955 Billingham et al. (21) showed that the injection of normal CBA lymph node cells into tolerant CBA mice could cause the rejection of a previously tolerated A strain skin graft. It wasn't until 3 years later, however, that the effect of incompatible immunocompetent grafts on the host itself were realized. Using similar transfer techniques Simonsen (207) set out to prove his concept of a GVH reaction by studying the effects of the intravenous inoculation of 18 day chick embryos with adult lymphoid cells. The developing chicks exhibited severe pathological changes including splenic enlargement to over twice that of normal chicks, and severe hemolytic anemia. Microscopic study suggested that the transplanted cells had been stimulated into multiplication and antibody formation by host antigens, and in so doing had largely replaced the native cell population.

From his first definitive study of the GVH reaction, Simonsen (207) put forth 3 basic criteria which are necessary for the clinical manifestation of a GVH reaction. These requirements remain valid today. The graft must first be comprised of immunologically competent cells capable of engaging in an immune response. Secondly, the host must

possess transplantation antigens foreign to the grafted cells and sufficiently immunogenic to elicit a response from them. A third criterion, which is necessary for complete expression of a GVH reaction, is that the host be incapable of mounting a countervailing immune response against the graft. Fulfillment of the first two conditions without the third will mean that a GVH reaction will take place, but its clinical expression will depend upon the strength of the counter-directed host-versus-graft reaction.

There are several experimental means by which the host's unresponsiveness to the graft can be insured. A generalized depression of the immunologic capacity of the host can be achieved by the use of X-irradiation (28), antimetabolites or noenatal thymectomy (2). Similarly, a GVH reaction can be initiated in a host that has been rendered specifically tolerant to tissue antigens of the donor by exposure of the host to these antigens at birth (2). Such methods of reducing responsiveness to the graft, however, interfere with the normal immune response of the host. By using genetic combinations of inbred strains it is possible to induce a strong GVH reaction in an adult host whose immune system is completely normal (73). An example of such a situation is the injection of parental strain lymphoid cells into F_1 hybrid offspring of a cross between the donor strain and an unrelated second strain. While the F_1 hybrid is unable to react immunologically to the histocompatibility antigens of either strain, the immunocompetent cells of the graft tissue can mount an immune response against the foreign antigens of the second parent expressed in the F_1 hybrid. The strength of the ensuing

GVH reaction can be pre-determined by varying the genetic disparity between the two parental strains.

The age of both the donor and recipient also seems to be a factor in determining the severity of GVH reactions induced by the injection of parental cells into F_1 hybrids. Younger animals, though immunologically mature, appear to be more susceptible to the effects of GVH reactions (73). The reason for this is not entirely clear, but it may reflect a greater susceptibility of younger organs to colonization by foreign cells, or a greater ability of older animals to detect subtle differences between donor and host cells. Similarly, graft cells from younger mature mice are more effective in eliciting a GVH reaction than cells obtained under the same conditions from older mice (37).

The GVH reactions previously described are of a systemic nature affecting the entire animal. In addition it should be pointed out that local GVH reactions are more easily induced intradermally and in areas such as underneath the renal capsule (66,74). In the case of local GVH reactions the immunologic status of the host is much less important, and much more information can be gathered from the reaction of the immunologically competent graft prior to its rejection by the host.

Pathology and Measurement.

Although the pathologic effects of both systemic and local GVH reactions vary from species to species, certain features are held in common (2,207,209). Growth retardation in newborns and wasting disease in adults are perhaps the most obvious effects of systemic GVH reactions.

It has been suggested that growth retardation is due primarily to loss of fat storage and lack of musculoskeletal growth. In addition, it has been shown that mitotic inhibition may occur in many tissues and organs during the course of GVH disease, and this may account for some of its pathological manifestations (45).

One of the most consistent manifestations of GVH disease is splenic enlargement. Splenomegaly has been observed in chickens, mice, rats and rabbits (209), and is one of the most common means of assessing the strength of a GVH reaction. The enlargement begins about 3 or 4 days after grafting and increases to a maximum size at about 8 or 10 days, after which it slowly declines. At the peak of splenomegaly, spleen sizes of 4 to 5 times normal have been reported. Outwardly, the enlarged spleens frequently appear paler than normal, but are just as often normal in both shape and color. Histologically, Howard (104) has shown a massive proliferation of pyroninophilic cells of the plasmacytic family. At the height of splenomegaly at least 50% of the total cell population was classified as plasmablasts or immature plasma cells. Malpighian follicles were completely destroyed within 7 to 9 days of the donor cell injection. At a later stage, macrophages have been shown throughout the spleen (241). In addition to phagocytosis of lymphocytes and plasma cells, a moderate number of erythrocytes are phagocytized and many macrophages contain pigments derived from hemoglobin.

In contrast to splenic enlargement, systemic GVH reactions generally cause marked involution of the other lymphoid organs (19,20,23). The

lymph nodes may pass through a temporary period of enlargement but the thymus does not (22). Within two weeks of GVH induction, the lymph nodes and thymi are greatly reduced in size, yellowish in color and hard in texture. Some have disappeared entirely. Much of this destruction of host lymphoid tissue may be the result of a non-specific cytotoxic effect which has been shown to be exerted against syngeneic, allogeneic and xenogeneic cells by lymphoid cells undergoing a GVH reaction (213).

Dermatitis and severe alterations of the skin are prominent features of GVH reactions in rats, and to a lesser extent in mice (20) Exfoliation is accompanied by thickening and loss of elasticity of the skin, which seem to be related to the infiltration of histiocytes and fibroblasts (23).

A wide range of hematological disorders have also been reported in cases of GVH disease. The most pronounced of these is severe anemia which can be attributed to both bone marrow hypoplasia (23,229) and production by the donor cells of antibodies directed against host erythrocytes (172,179). Lymphopenia is also a consistent finding in GVH disease.

A number of other organs are affected but their roles in the development of the GVH reaction are probably of only secondary importance. Severe diarrhea is very common, as is liver enlargement. Extensive cellular infiltration is frequently seen in the omentum and pancreas, and in terminal cases inflammatory changes are visible in the lungs (88).

Not surprisingly, the severity of pathological changes is proportional

to the strength of the ongoing GVH reaction. Since many of these changes can be expressed quantitatively they provide a means of measuring the extent of a given GVH reaction. The more obvious effects such as mortality rate and body weight change have been used to estimate GVH severity (196), but host related and envrionmental variables frequently make these methods unreliable. In cases in which the host has been immunosuppressed, the complex interaction of irradiation or drugs with the GVH reaction itself makes it difficult to attribute mortality and weight loss specifically to the GVH reaction.

Perhaps the most commonly used assay for GVH intensity is the spleen weight assay developed by Simonsen (106,209,212). The ratios of spleen weight to body weight are determined for GVH experimental animals and normal littermate controls. GVH activity is then expressed by the "spleen index" which represents the relationship of spleen to body weight for GVH mice relative to that for littermate controls. A spleen index of 1 would therefore represent a normal spleen while an index of 2 would be indicative of considerable splenomegaly.

Although there is a linear relationship between the spleen index and the logarithm of the dose of inoculated lymphoid cells, the splenic enlargement is largely due to the proliferation of host cells (106). Thus the spleen index reflects not only the immunologic potency of the donor cells, but host responsiveness to proliferative and inflammatory stimuli as well. Care must therefore be taken in making a comparison between spleen indices of hosts with different ages, sex, genotype etc.

Furthermore, it should be remembered that host proliferative responses such as splenomegaly do not play an essential role in the pathogenesis of GVH disease (102).

A liver weight assay similar to that for the spleen has also been described. Since the liver enlargement produced by a GVH reaction is invariably much less than that produced in the spleen, however, liver assays are much less sensitive than spleen assays and subject to the same drawbacks.

A phagocytosis assay has been developed by Howard (104) based on his findings that intravenously injected colloidal carbon is cleared from the blood by the reticuloendothelial system at an increased rate during the course of a GVH reaction. The advantage of this test is that quantitative data can be obtained without sacrificing the animal The specificity of this assay is questionable, however, since there is no way to distinguish between increased phagocytosis resulting directly from the GVH reaction and that resulting from complications such as infection and inflammation.

An assay of graft-host interaction has been described by Miller et al. (151) based on their finding of liver infiltrates of donor origin following the injection of competent tissue in the mouse. Enumeration of microscopic foci along the branches of the portal vein was used as an indication of donor cell activity. A number of reports, however, have shown that such perivascular infiltrates may also develop as non-specific lesions, often associated with abdominal inflammation and certain systemic infections such as mononucleosis (178,195). This assay, like the

spleen index therefore, need not represent a direct indication of GVH activity.

In addition to the systemic reactions previously described, local GVH reactions are frequently produced experimentally by the injection of immunocompetent cells intradermally or under the renal capsule (66,74). In such conditions the pathogenesis of the GVH reaction can be studied more directly while the concomitant immunologic response of the host is greatly reduced. The intensity of these reactions can be measured by subjective evaluation of induration, erythema and vascular permeability (67). Ford et al. (75) have found that the popliteal lymph nodes draining an area undergoing a local intradermal GVH reaction show weight increases up to 30 times that of control nodes. They have developed a quantitative assay of local GVH activity based on this increase in lymph node weight.

The use of particular assays in evaluating GVH intensity depends upon the experimental conditions Since each measures a different facet of the GVH reaction and each has its inherent weaknesses, several of these assays should be considered in determining the activity of a given GVH reaction.

Antigens Responsible for GVH Reactions.

As with most transplantation reactions, the intensity of a GVH reaction is determined by the disparity of histocompatibility antigens expressed by the donor and the host. Incompatible LD and SD antigens expressed on the host tissue and not present on the donor cells will

elicit a GVH response. Although H antigens expressed by the donor cells are also important, the experimental selection of an unresponsive host will nullify their effect.

As described earlier, each species has a major H locus responsible for the production of "strong" transplantation antigens. These major loci are in the mouse, H-2; in the rat, Ag-B (H-1); the chicken, B; and man, HL-A. GVH reactions can readily be elicited by one of these strong antigens without prior specific immunization of the donor. In the mouse, for example, a single H-2 antigen can stimulate a GVH reaction with sufficient intensity to produce significant splenomegaly in the host. In the absence of H-2 disparity, however, splenic enlargement is not readily elicited (63), and preimmunization of the donor or a higher dose of donor cells is usually necessary (209).

Until recently, it was thought that the genetic requirements for a GVH reaction were the same as those for skin graft rejection, e.g. H-2K and H-2D incompatibility. Recent delineation of the H-2 locus, however, has shown that GVH reactions are stimulated most efficiently when there is Ir region incompatibility, but only weakly by H-2K, H-2D and Sd-Slp incompatibility (173). In fact, Livnat and Klein (131) have shown that GVH reactions can occur in combinations of strains which are serologically indistinguishable and which accept each other's skin grafts permanently. The requirements for GVH reactivity, therefore, have been shown to closely resemble those for the MLC reaction (57,189).

The strength of the major H antigens may be largely due to the high frequency of lymphocytes which can be stimulated by these antigens (210).

Support for this concept comes from the fact that it is often difficult to obtain a GVH reaction across species barriers, even in cases where the host environment permits the survival and immunologic function of the donor cells (67,124). In such cases it is believed that there is a much lower frequency of cells capable of recognizing xenogeneic antigens than capable of recognizing the major H antigens on allogeneic cells

Cell Interactions in GVH Reactions.

Although antibodies directed against host tissues have been identified in animals undergoing such reactions (172,179), GVH reactions have been shown to be primarily cell-mediated immune responses (7,209). Some studies have suggested that a cell cooperation similar to that observed in antibody formation is an essential part of these reactions. Early experiments with irradiated F_1 hybrid mice of parent strains differing at the H-2 locus, suggested that neither parental T lymphocytes nor B lymphocytes alone can initiate a GVH reaction sufficient to produce significant splenomegaly (12,27,234). An inoculum of half the cell dose but containing both T and B lymphocytes, however, produced a severe splenic enlargement. Splenomegaly, however, may have non-immunologic causes and is not always a good indicator of GVH activity (102). For example, the splenomegaly observed in these experiments could easily result from a general repopulation of lymphoid cells in the irradiated host rather than a synergistic effect between T and B cells in eliciting a GVH reaction. Splenic enlargement has also been shown to be caused by inflammatory responses (65,67) as well as non-immunologic proliferation

of bone marrow cells (103). Because of this, many authors (55,67,231) refute the concept of thymus-bone marrow synergism in GVH reactions.

The reactive cells initiating GVH reactions were first identified by Gowans (90) as recirculating, long-lived, small lymphocytes of the type found in the thoracic duct. Although Gowans' studies suggested that these cells were mature T cells, more recent studies by Cantor and Asofsky (7,36,37) suggest that a synergistic relationship may exist between two populations of thymus-derived cells. The addition of normally unreactive young thymus cells to a lymph node or spleen suspension has been shown to greatly enhance their ability to induce a GVH reaction (35,37). The less mature cell type found in the thymus is relatively unaffected by anti-thymocyte serum (ATS). It appears to determine the specificity of the response and is probably the precursor of effector cells. The more mature T cell found in the spleen is highly sensitive to ATS and is believed to amplify the response of the effector cells (36).

A recent report by Argyris (5) supports the concept of two T cell populations being necessary for GVH activity and shows that macrophages may also contribute to the GVH reaction. Treatment of C57BL/6 donor spleen cells with anti-θ serum or anti-macrophage serum decreased the GVH activity in newborn C3H x C57 F_1 mice. Treatment of donor cells with anti-B serum, however, had no effect on GVH activity, suggesting that B cells are not essential for expression of GVH reactivity.

In his early studies on the GVH reaction, Simonsen (207,212) reported that host antigens stimulated donor lymphoid cells to multiply and form antibody, thereby destroying and replacing the host cell population.

This theory was later challenged by Fox (76,77) who used chromosome marker techniques to show that extensive donor cell proliferation is not a constant feature of the GVH reaction. When CBA (T6) spleen cells were used to induce GVH reactions in CBA x C57BL F_1 adult recipients, three consecutive phases of cell proliferation were observed in the host spleen: 1) a tremendous early burst of donor cell proliferation accounting for up to 60% of cell mitoses but lasting only 48 hours; 2) a more protracted rise of host mitotic activity resulting in splenomegaly; and 3) a final period when donor mitotic activity was constant but accounted for only about 1% of cell mitoses. Similar results were observed when GVH reactions were initiated by the injection of adult CBA spleen cells into one day old A strain mice (246). In both cases, no donor cell proliferation was seen in the host bone marrow.

Subsequent studies showed that donor cell repopulation was more extensive when the GVH reaction was more severe (77,78). When C57BL spleen cells or CBA spleen cells which had been presensitized to C57BL were injected into adult CBA x C57BL recipients, the same initial burst of donor cell proliferation was observed in the spleen as had been earlier observed with CBA cells With C57BL cells, however, the proportion of donor cell mitoses continued to increase rather than decline. At the time of maximal splenomegaly on the thirteenth day all mitoses were of donor origin. By the third week post GVH induction, 70% of mitoses in the bone marrow were of C57BL donor origin.

The above findings suggested that the extent of donor lymphoid cell proliferation in the host spleen depends upon the strength of antigenic

stimulation presented to the donor cells by the host tissue. Strength of stimulation, in turn, is determined by such factors as genetic disparity between donor and host and by prior sensitization of the donor to host antigens. In addition, it was later found that the immunological status of the host is also an important factor governing the extent of donor cell proliferation. Prior irradiation and thymectomy of the host reduces the degree of splenomegaly (102,103,106), but leads to an increased proliferation of donor cells (78,106) and an increased severity of the GVH disease. These observations and similar results obtained in our laboratory (233) suggest that the splenomegaly observed in GVH animals results primarily from a proliferation of host cells which may in turn protect the host from the lethal effects of the GVH reaction.

Donor lymphoid cells were originally thought to retain their immunologic activity against host tissue antigens throughout their residence in the host spleen. This assumption was based on early experiments in which GVH reactivity was serially transferred in newborn mice (214) and outbred chickens (207). These experiments were not well controlled, however, and subsequent studies (78,106,208) using discriminant spleen assays showed that GVH reactivity could be serially transferred to hosts of a new strain combination but not to hosts of the same F_1 combination as the original hosts. It was concluded from this that donor lymphoid cells become specifically tolerant to host tissue antigens while retaining immunological reactivity against other transplantation antigens.

The frequent remission of GVH symptoms and subsequent survival

of many GVH mice has often been attributed to the acquisition by donor cells of tolerance to host antigens (78,106,208). Subsequent studies have shown that this may not be the case. Fox and Howard (79) discovered that adult F_1 mice which survived an acute GVH syndrome were highly resistant to a second inoculation of reactive lymphoid cells from either parental strain. This acquired refractoriness could not be transferred passively with serum or adoptively with host spleen cells to a secondary host. A similar refractoriness was observed in rats by Field and Gibbs (72). In these experiments parental type skin grafts were accepted by resistant F_1 hosts, suggesting that the hosts had not become immunized to the donor cells. Parabiosis for a limited period during the active stage of the disease was sufficient to confer resistance upon a new, previously susceptible F_1 host (70). Furthermore, irradiation of the blood extracorporally during cross-circulation did not prevent the transfer of refractoriness. From these findings it was postulated that the refractory state probably resulted from the production in the first host of an inhibitory factor such as a blocking antibody.

An intriguing alternate explanation for GVH-refractoriness was presented by Ramseier and Lindenmann (185) who showed that, while F_1 hybrids cannot react to parental H antigens they can form antibodies against recognition structures which their hybrid cells specifically lack. Adult F_1 hybrid animals from inbred strains of rats, mice and Syrian hamsters were immunized with lymphoid cells from one of the

parental strains. In all three species an activity appeared in the serum of treated hybrids which specifically prevented cells of the immunizing parent from recognizing transplantation antigens of the other parent present on F_1 hybrid cells.

Such a theory as proposed by Ramseier and Lindenmann could explain the GVH refractoriness observed by Fox and Howard and by Field and Gibbs. Some question has been raised, however, as to the ability of parental receptor sites to generate an immune response by the F_1 host sufficient to inhibit parental cell activity. Although this may account in part for the inhibition of donor cell activity, recent evidence suggests that much of this is also due to a general loss of immunological responsiveness by animals undergoing a GVH reaction (95).

Effects of the GVH Reaction on Immunologic Responsiveness.

Studies of the response of animals experiencing GVH reactions to other antigens have resulted in reports of both depressed and enhanced immunologic function caused by the GVH reaction Although by far the most consistent finding has been immunosuppression, a brief description of cases showing increased responsiveness is in order.

Reports of increased immunologic function during a GVH disease have centered around the response of such animals to certain intracellular pathogens. In 1961 Cooper and Howard (46) reported that the mortality rate from a bacteremic infection with Diplococcus pneumoniae was reduced from 80% in normal adult F_1 mice to nil in littermates undergoing the early stages of GVH reactions. They attributed this

increased survival to phagocytic hyperactivity of the reticuloendothelial system brought about by the early stages of the GVH reaction.

In a similar series of experiments Blanden (26) demonstrated an increased resistance on the part of GVH mice to infection by Listeria monocytogenes and Salmonella typhimurium. He showed that this resistance resulted from an activation by the GVH reaction of macrophages in the host spleen, liver and peritoneal cavity. These macrophages exhibited a greatly increased ability to inactivate Salmonella and clear it from the blood. This effect may be closely related to the previously described non-specific cytotoxicity of lymphoid cells undergoing a GVH reaction (213). It is significant that, while the cellular resistance of these animals to bacterial infection was increased, their humoral response to sheep red blood cells was completely suppressed. Similarly, Shevelev (201) reported a marked depression of antibody synthesis to Salmonella in animals undergoing a GVH reaction. Under similar experimental conditions, Howard (105) had reported an increased sensitivity in GVH mice to purified endotoxins. These seemingly contradictory findings are not as inconsistent as they might first appear.

In addition to increased resistance to certain intracellular parasites, animals undergoing GVH reactions have exhibited striking increases in the production of antibodies to certain hapten-carrier complexes. Katz et al. (114,115) sensitized CAF$_1$ mice to a complex of 2, 4 dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) and injected them 14 days later with spleen cells from normal parental A strain donors. Following a subsequent challenge with DNP-KLH, the mice which received allo-

geneic cells exhibited an anti-DNP antibody response which was up to 50 times greater than the response of normal mice receiving only the 2 injections of DNP-KLH. This phenomenon has been termed the allogeneic effect , and has been shown to depend upon the presence of an ongoing GVH reaction in the test animals. It has been suggested that this phenomemon results from the production by GVH reactive cells of non-specific T cell-secreted mediators with the capacity to stimulate B cells to antibody formation following their interaction with antigen. The degree to which this phenomenon influences antibody production is related to three important factors: 1) prior sensitization of the host, 2) the intensity of the GVH reaction induced, and 3) the time interval between allogenic cell transfer and secondary antigenic challenge. These factors are crucial, since a suppression of the antibody response was observed if a strong GVH reaction was induced, or if the time interval was too long before secondary challenge with DNP-KLH (174).

Immunosuppressive Effect

Suppression of both humoral and cell-mediated immune responses has been consistently reported in animals undergoing a GVH reaction. In 1961 Howard and Woodruff (107) first showed that allograft rejection can be suppressed by the GVH reaction. They initiated a weak GVH reaction in C57BL x CBA F_1 mice by the injection of CBA lymphoid cells and found that subsequent A strain skin grafts showed normal first- and second-set rejection times. When a strong GVH reaction was produced by injecting C57 lymphoid cells into C57 x CBA F_1 mice, these animals

showed delayed rejection of both first- and second-set A strain skin grafts.

Lapp and Møller (125) later found that when H-2 incompatible skin grafts were given to F_1 mice on the same day as the injection of parental lymphoid cells, there was no prolongation of graft survival. If the skin grafts were given at least 7 days after the parental lymphoid cells, however, significant prolongation of graft survival was observed. Furthermore, sensitizing the F_1 animals to third party skin before the induction of the GVH reaction caused an even greater prolongation of the survival of subsequent allografts from the same third party.

Suppression of the humoral immune response by the GVH reaction has been studied in greater depth than suppression of the cell-mediated response. The effect of the GVH reaction on the primary response of both host and donor cells to SRBC has been studied both *in vivo* and *in vitro* (26,54,64,127,159,215,244) and a summary of these observations can be made for moderately strong GVH reactions. When the prospective hosts were sensitized to SRBC 2 days before the induction of the GVH reaction, the resulting number of antibody-producing plaque forming cells (PFC) was no different from control values. When SRBC were injected into the donor at the same time as the donor lymphoid cells there was frequently an increase in the PFC response over control values. If however, the host received its first injection of SRBC more than 3 days after induction of the GVH, there was a marked depression in the PFC response to SRBC. Maximum suppression of the humoral response to SRBC was achieved at 7 days post GVH induction and continued thereafter.

Suppression of the humoral response was also observed when donor cells presensitized to SRBC were used to initiate a GVH reaction. Lawrence and Simonsen (127) injected presensitized parental cells into irradiated recipients and found that the transferred parental cells lost their ability to produce antibodies against SRBC after 7 to 10 days' residence in the hybrids. Möller (159) then transferred presensitized parental lymphoid cells into untreated F_1 hybrid hosts and found that the fate of the parental antigen sensitive cells as well as antibody forming cells depended upon the genotype of the F_1 host. When the donor parental cells were of H-2^b genotype (C57BL) there was a marked decrease in both the number of antigen sensitive cells and PFC in the host. When the parental cells were of H-2^a (A) or H-2^k (CBA) genotype, however, the PFC response frequently increased.

In addition to the numerous experiments with SRBC, GVH reactions have also been shown to suppress the humoral response to bovine serum albumin (119), keyhole limpet hemocyanin (26), T_2 bacteriophage (25), thymus specific antigens (245), and a number of bacterial endotoxins (159,201). Suppression has been reported to be as complete with thymic-dependent antigens (159).

A quantitative study of normal serum immunoglobulin levels has been made in animals experiencing GVH reactions (119). By the 14th. day post-GVH induction, the levels of circulating IgM, IgG and IgA were found to have decreased 50% or more in most mice studied. Immunization with BSA caused an increased level of all three immunoglobulins in

control mice while the level of IgG and IgA continued to fall in GVH mice. The IgM level in GVH mice did increase somewhat following stimulation with BSA, but it never reached control values.

The degree of immunosuppression brought on by a GVH reaction can be seen to depend upon several factors: 1) the strength of the GVH reaction as determined by histocompatibility differences and the dose of donor cells 2) the source of the donor cells, and perhaps most important 3) the duration of the GVH reaction at the time of immunization. F_1 recipients are fully competent to produce antibodies and immunosuppression will not occur until the parental cells have resided in the hybrid for more than three days. An unfavorable host environment could result from a non-specific stress response on the part of the host to the immunologic attack upon his lymphoid system. A resulting higher level of circulating steroids might cause a general suppression of the immune response.

Early investigations (25,26,72,107,213) suggested that the GVH reaction may bring about immunosuppression by the immunologic attack of competent donor cells on the lymphoid cells of the recipient. This would cause a destruction of host lymphoid tissue leading to a depletion or inactivation of cells capable of mounting an immune response. More recently GVH induced immunosuppression has been explained in terms of antigenic competition, but since the mechanisms causing antigenic competition are equally as vague this does little to clarify the situation. Antigenic competition, in turn, is believed to result from one or a combination of three factors (215): 1) a specific competition of two

possibly cross reacting antigens for the same antigen sensitive cells, 2) a non-specific competition for limiting factors such as space, nutrients, etc. and 3) the production of inhibitory factors by the initial immunologic reaction.

Lawrence and Simonsen (127) proposed that cells involved in the GVH reaction competed with other antigens for pluripotent cells capable of reacting with them. By virtue of its much greater intensity the GVH reaction was thought to take up cells and space for reactions with other antigens. Somewhat later, Lapp and Möller (215) found that adoptive transfer of normal parental or F_1 lymphoid cells into an F_1 (GVH) animal does not restore immunocompetence. These results suggested that the environment in the hybrid host is responsible for immunosuppression. Work by Lapp, et al. (126) has shown that immunocompetence can be restored in GVH mice by the injection of crude thymic extracts from syngeneic mice. It appears, therefore, that at least part of the immunosuppression brought on by the GVH reaction results from the depletion of a factor found in limited amounts in the normal thymus (95).

The in vitro work of Sjöberg (215,216) and the in vivo studies of Möller (159) suggest that an inhibitory factor may also be produced by cells undergoing a GVH reaction. Recent evidence suggests that macrophages may play an important role in the production of such a substance (33,64,215). This substance could cause inhibition by interfering with normal cooperation between T cells and B cells or by suppressing the proliferation and recruitment of cells from the bone marrow. In addition, Elie et al. (64) have found evidence that a soluble factor pro-

duced by T cells can restore the response of GVH spleen cells to SRBC in culture. They have proposed a feedback type of mechanism to explain the causes of GVH induced immunosuppression. They have proposed that both T cells and macrophages produce soluble factors which interact to form a complex. B cell activation would require the binding of this complex as well as antigen, a concept in agreement with studies showing a requirement for non-adherent cells in normal primary and secondary immune responses (29). An excessive amount of uncomplexed macrophage factor could produce a negative feedback to turn off T cell factor release or inhibit B cell activity. Using this model, the initial immunosuppression observed in GVH animals could be explained by a depletion of T cell factor by cells responding in the GVH reaction The long lasting immunosuppression could result from feedback inhibition of T and B cells resulting from the increased macrophage activity known to occur in GVH animals. Although this proposal is highly speculative, it provides a means of bringing together many recent findings into a single workable model.

Significance of the GVH Reaction as a Research Tool.

The direct clinical applications of the GVH reaction are manifold. Its most obvious clinical application will be the eventual ability to control GVH reactions and thus permit the successful grafting of healthy immunocompetent cells into patients suffering from aplasia or neoplasia of their own lymphoid cells. In addition, its control might

have important ramifications in clinical transplantation where local GVH reactions have been implicated in the rejection of transplanted organs (96) Since the GVH reaction might be considered as an autoimmune disease, a clearer understanding of it will lead to increased control of autoimmune diseases such as hemolytic anemia and rheumatoid arthritis.

The GVH reaction represents a unique situation in which the most complex of cell interactions and immunological principles are all brought together in one reaction. It provides a unique opportunity to study these principles and interactions together within one experimental model. By studying the GVH reaction it may be possible to identify and elucidate those factors that regulate both cell-mediated and humoral immunity. This in turn could lead to the ability to control or manipulate the immune response for application in specific clinical situations. This thesis therefore presents a study of the regulation of cell-mediated and humoral immunity in animals experiencing a GVH reaction.

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CHAPTER 2: MATERIALS AND METHODS

Animals

Mice of inbred strains A ($H\text{-}2^a$), CBA ($H\text{-}2^k$), C57/BL6 ($H\text{-}2^b$) were used as the parental strains in these experiments. Two F_1 hybrids derived from these strains, i.e. CBA X A and C57/BL6 X A, were also used. For simplicity, C57/BL6 will be referred to as B6, C57 BL6 X A will be termed $B6AF_1$, and CBA X A will be called CAF_1.

All mice used in the present study were from a closed colony which has been maintained in this laboratory for over 10 years. Syngenicity was ensured by strict brother-sister matings and by using animals which were never more than 4 generations removed from a common brother-sister mating. The syngenicity of each inbred strain was verified at regular intervals using the "ring test" procedure described by Billingham and Medawar (2).

Unless stated otherwise, the donors and recipients used for the GVH reaction and for skin grafting were mature males 12-14 weeks of age. Mice used as bone marrow and kidney donors were males 2-4 weeks of age.

Antiseptic Technique for Operating Procedures

All operations in these experiments were performed under antiseptic but not sterile conditions. Surgical instruments were boiled in a steriliser for 30 minutes, and all glassware used in the operations was heated to 150°C in a dry oven for 1 hour before the operation. Cork surgical

boards and adjacent table tops were washed with a 70° ethanol solution containing 1 "Cetavlon". Prior to operating, the hands and forearms of the operator were washed thoroughly with "Hexaderm", an antibacterial detergent containing 3% hexachlorophene. Surgical gloves were not used. The hands of the operator did not touch the animal during surgery, and all contact with the animal was made with sterile instruments.

Skin Grafting

Full thickness skin grafts were applied using the technique described by Billingham and Medawar (2) with modifications introduced by Bliss (3). Donor mice were killed by cervical dislocation, after which they were shaved and their skin washed with a 70% ethanol solution. "Pinch grafts" were taken from the mid-dorsal area, avoiding areas of new hair growth. Grafts were cut by raising the cleanly shaven skin into a cone with fine forceps and cutting an oval piece of skin with fine curved scissors. The grafts were placed epidermal side down on a sterile filter paper soaked with physiological saline in a covered petri dish. To ensure proper healing, fatty and connective tissue was removed from the underside of each graft using curved Metzenbaum scissors.

Recipient mice were anesthetized in an ether chamber and stretched out on their sides and secured to a cork board by means of rubber bands attached to the front and back legs. The lateral chest wall was then shaved, clean and washed with a 70% ethanol solution. The graft bed was prepared by removing a pinch graft from this area in the same manner

which was used to prepare donor grafts. When second-set grafts were being made, they were placed on the opposite side of the chest from the site of the first-set grafts. Care was taken to avoid damaging the lateral thoracic and mammary blood vessels which provide a good blood supply to the healing graft. The donor graft was placed in the prepared graft bed and dusted lightly with sulphadiazine powder. A 4 inch strip of 3/4 inch width "Scotch Magic Transparent Tape" was then wrapped around the thoracic cage to hold the graft in place

Graft dressings were removed on the 7th post-operative day and grafts were visually inspected daily. The grafts were scored from 0 to 100 based on the amount of healthy epithelium still surviving. Grafts with 10 per cent or less healthy epithelium were considered to be rejected on the following day.

Adrenalectomy.

Each mouse was anesthetized in an ether chamber and placed on his left side on a cork board. A small cotton swab was soaked with ether and placed by his nose to maintain the proper level of anesthesia. Hair was shaved from a lateral area just below the ribs and the skin was washed with a 70% solution of ethanol. A small incision about 5 mm in length was made in the lateral abdominal wall just below the last rib. The abdominal wall was retracted by inserting a pair of fine forceps and allowing them to open. Taking care not to damage the renal vascular supply, the right adrenal gland was located in the fatty tissue just

above the kidney. The right adrenal gland was isolated using a pair of fine, curved forceps, and the gland was either "teased" free with the forceps or delicately cut free with fine scissors. When surgery is done carefully in this manner the adrenal gland can be removed without any loss of blood. Following removal of the adrenal gland, the abdominal muscle and skin incisions were closed separately using interrupted sutures with 5-0 surgical silk. Sulfadiazine powder was sprinkled lightly over the wound and the animal was then placed on his right side for the removal of his left adrenal gland in a similar manner. Following adrenalectomy the mice were allowed a choice of tap water or a 0.5% solution of NaCl ad libitum. At the conclusion of the experiments each mouse was sacrificed, and a post mortem examination was performed to confirm complete removal of the adrenal glands.

Castration.

Each mouse was anesthetized in an ether chamber and placed on a cork board in a supine position. Cellophane tape was placed over the hind legs to maintain this position. The scrotal area was shaved clean and washed with a 70% solution of ethanol. A small incision of about 10 mm was made in the mid-scrotum. The common vaginal tunic was cut open with a pair of fine scissors and the testes were exposed by pressing gently on the abdomen. Using 3-0 surgical silk, a ligature was placed around the spermatic cord to include the testicular artery and vein. The testis was then removed by cutting the spermatic cord and testicular

vessels about 1 mm distal to the ligature. The other testis was then removed in the same manner, after which the common vaginal tunic and scrotum were closed using interrupted sutures with 5-0 surgical silk. The wound was then dusted lightly with sulphadiazine powder.

Induction of GVH Reactions.

Strain A mice were sacrificed by cervical dislocation, and the lymph nodes and spleen were removed for the preparation of a lymphoid cell suspension (LC). To remove the lymph nodes and spleen the animal was placed on his back and the entire ventral surface was washed with a 70% ethanol solution. A longitudinal incision was made along the ventral midline from the mandible to the pubis. Transverse incisions were then made at each end of the longitudinal incision, leaving two flaps of skin which were drawn back to expose the inguinal, brachial, axillary and cervical lymph nodes. The lymph nodes were removed and placed in cold Earle's balanced salt solution supplemented with 10% heat inactivated calf serum (BSS). To remove the spleen, the animal was then placed on its right side, an incision was made through the abdominal muscle, and the spleen was removed and placed in BSS.

Lymphoid cell suspensions were prepared by gently pressing the lymph nodes and spleen through a #50 mesh stainless steel screen into BSS. The cells were washed three times in BSS by centrifugation at 700 G for 10 minutes. After the third washing the cells were strained through sterile gauze to remove any clumps which had formed. The cells were

then counted using the trypan blue dye exclusion technique, and resuspended in BSS to give a concentration of 250 X 10^6 cells per ml. The F_1 recipients were warmed under an infra-red lamp to dilate the tail veins, and the cells were injected via the tail vein in doses of 0.3 ml containing 75 X 10^6 living cells. A tuberculin syringe and a 26 guage needle were used for the injections.

Preparation of Antigens.

Bone marrow (BM) cells were obtained from B6 and CAF_1 femora and tibiae. The mice were killed by cervical dislocation. The head of each bone was cut off and BM cells were aspirated with a 26 guage needle and collected in BSS. The cells were injected intravenously in dosages of 40 X 10^6 cells per recipient (10).

Kidney cell suspensions were prepared by mincing B6 donor kidneys and gently pressing them through a #50 mesh stainless steel screen. The cells were suspended in BSS and injected intraperitoneally in a volume of 1.0 ml to give an equivalent of one-half kidney per mouse.

B6 red blood cells (B6 RBC) and CAF_1 red blood cells (F_1 RBC) were collected by cardiac puncture using an acid-citrate-dextrose solution as an anticoagulant. The animal was first anesthetized with ether and placed in a supine position. After being washed with a 70% solution of ethanol, the skin was cut in a V-shape manner from the xipho-sternum to each shoulder. The flap of skin thus created was then lifted, and a longitudinal cut was made through the sternum. The chest wall was retracted

to expose the beating heart, and blood was withdrawn using a tuberculin syringe and a 22 guage needle inserted into the right ventricle. The syringe and needle contained a small amount of acid-citrate-dextrose solution to prevent coagulation

Sheep red blood cells (SRBC) were prepared from a solution containing a 50:50 ratio of sheep blood and Alsever's solution obtained from the Institut de Microbiologie et Hygiène, Laval des Rapides, Quebec. The sheep blood solution was washed twice with isotonic saline and centrifuged at 700 G for 10 minutes. They were then resuspended in saline to a concentration of approximately 16.7 X 10^8 SRBC/ml. The standard dose for immunization was 5 X 10^8 SRBC in aliquots of 0.3 ml when injected into the tail vein, and 7 X 10^8 SRBC in aliquots of 0.5 ml when injected intraperitoneally.

Preparation of Lipopolysaccharide.

The lipopolysaccharides (LPS) used in these experiments were obtained from Difco, Detroit, and were extracted from E. coli 055:B5 by the Westphal method. The method used for preparing the LPS was essentially the technique described by Müller (8). A solution containing 1 mg of LPS per ml of saline was heated in a double boiler at 100°C for 1 hour. In some instances mice were immunized directly with this solution, but in most cases the LPS was coated to either SRBC ~~or CAF~~$_1$ RBC which had been prepared as described above. In order to coat RBC, the LPS solution was allowed to cool and mixed with an equal volume of either packed SRBC or packed F_1 RBC. The RBC were incubated in the LPS solu-

tion for 45 minutes at 37°C and were gently swirled at regular intervals to keep the cells suspended. The RBC were then washed 3 times in isotonic saline, with centrifugation at 700 G for 10 minutes. The packed SRBC coated with LPS (SRBC-LPS) and the CAF_1 RBC coated with LPS (F_1-LPS) were both resuspended in saline to a final concentration of approximately 16.7 X 10^8 RBC/ml.. In all cases the SRBC-LPS and F_1-LPS were injected intraperitoneally in doses of 7 X 10^8 RBC contained in 0.5 ml of saline.

Delayed Hypersensitivity Assay.

The procedure followed for the induction and measurement of delayed hypersensitivity reactions to SRBC was a modification of the method described by Axelrad (1). Sensitization to SRBC was achieved by means of an intradermal injection of an emulsion of SRBC in complete Freund's adjuvant (CFA). To prepare this emulsion, a 10% (V/V) suspension of SRBC in saline was made in the same manner used for intravenous sensitization to SRBC. Equal volumes of SRBC in saline and CFA were emulsified immediately before injection. Emulsification was done by placing 1 ml of SRBC and CFA in separate glass syringes. The syringes were then connected by means of a common tube with luer-lok fittings on each end. The contents of the 2 syringes were drawn back and forth between the 2 syringes for 15 minutes until a uniform emulsion was formed. Each recipient mouse was injected intradermally with 0.01 ml of this emulsion in the right footpad using a 26 guage needle.

The sensitized animals were challenged with SRBC 9 days after the initial sensitization with SRBC. A 10% (V/V) suspension of SRBC in saline was prepared as described above. Each mouse was injected intradermally with 0.01 ml of the SRBC in saline in the left footpad using a 26 guage needle. An interval of 20 hours was allowed for maximum development of a delayed hypersensitivity response.

Measurements of the volume of the left foot were made immediately before the challenge with SRBC and again 20 hours after challenge. The method used for measurement of foot volume was based on the principle that an object immersed in a fluid displaces its own volume. If the object has a lower specific gravity than that of the fluid, the pressure needed to achieve total immersion will be proportional to the volume of the object (320). A Mettler P-1200 top-loading, single pan balance of 1200 g capacity with an optical scale calibrated in 100 mg divisions was used. A 5 ml beaker was filled with mercury and the scale was adjusted to a baseline of 0 with the beaker of mercury on the weighing pan. A line was drawn on the mouse's paw in the groove immediately distal to the lateral malleolus and the foot was immersed in the mercury with the experimenter's hand resting on a firm bridge just above the balance. The pressure required to immerse the paw was noted for 3 separate measurements, and the average of the 3 measurements was taken. Measurements were made on mice chosen at random and without knowledge of the experimental group from which they were taken.

In each experiment a group of unsensitized control mice were

challenged with SRBC and the amount of footpad swelling was noted. The amount of non-specific swelling in these control mice never exceeded 2% of the initial paw volume. The mean amount of swelling for the unsensitized control group was determined in each experiment and served as the basis for the measurement of the delayed hypersensitivity response. Delayed hypersensitivity (DH) was calculated as the per cent increase in paw volume for each mouse according to the following formula:

$$DH = \frac{PE_2 - PE_1}{PE_1} - \frac{PC_2 - PC_1}{PC_1} \times 100$$

where PE_2 is the pressure required to immerse the experimental foot after challenge;
PE_1 is the pressure required to immerse the experimental foot before challenge;
PC_2 is the mean pressure required to immerse the control feet after challenge;
PC_1 is the mean pressure required to immerse the control feet before challenge.

Detection of Antibody Forming Cells.

The method used for assaying the total number of direct plaque forming cells (PFC) to SRBC was essentially the one described by Cunningham and Szenberg (4) with slight modifications (6). The spleens were removed from sensitized and control animals in the manner described for the preparation of lymphoid cell suspensions for the induction of GVH reactions. Each spleen cell suspension was prepared by gently pressing the spleen through a #50 mesh stainless steel screen into BSS. The

cells were then strained through the mesh again, and the screen was rinsed with BSS to obtain as many spleen cells as possible. BSS was added to the cell suspension to a final volume of 15 ml. Cell suspensions were maintained at 4°C during preparation and up to the time of assay.

Target cells for the PFC assay were either SRBC, SRBC-LPS or F_1-LPS. The F_1 RBC and SRBC were obtained and coated with LPS as described in the procedure for sensitization to LPS. The SRBC, SRBC-LPS, or F_1-LPS were washed twice in isotonic saline with centrifugation at 700 G, and resuspended at a final 10% concentration in saline. Guinea pig complement (Grand Island Biological Co., Grand Island, New York) was diluted in BSS to a 10% concentration.

The PFC assay was performed by mixing 0.05 ml of spleen cells, 0.15 ml of RBC target cells, and 0.75 ml of the complement solution in a test tube at 37°C. The mixture was withdrawn with a Pasteur pipette and put into chambers made by glueing two 75 X 25 mm microscope slides together with double-sided tape. Six slide chambers were required for each sample. If necessary, filling of the last chamber was completed using a "blank" solution of target RBC and complement in the same concentrations as for the test mixture. The slide chambers were sealed with warm paraffin wax, and incubated at 37°C for 1 hour. The identity of PFC was confirmed with microscopic examination, and the number of PFC were then counted macroscopically. The total number of PFC for each spleen was estimated by multiplying the total number of PFC counted for all chambers by 300. The factor of 300 represents

the proportion of spleen cells used for the assay, ie. 0.05 ml of spleen cells for a total suspension volume of 15 ml.

The same protocol was followed for the development of indirect plaques except that rabbit anti-mouse IgG antiserum (Pentex, Miles Laboratories, Kankakee, Illinois) was added to the solution of BSS and complement to give a final antiserum dilution of 1:200.

The direct PFC assay was used to detect the presence of cells forming IgM antibody to the target RBC while the indirect assay detected cells producing both IgM and IgG antibodies. The number of cells forming IgG antibody can then be determined by subtracting the direct PFC count from the indirect count.

Detection of hemagglutinating antibodies.

Determinations were made of the levels of hemagglutinating antibodies to SRBC, SRBC-LPS, F_1-LPS, and B6 RBC. The target RBC for the hemagglutination assay were obtained and/or labeled as described for the sensitization procedure.

The method used to determine the amount of circulating antibody to each antigen was a modification (11) of that described by Kaliss (5) using polyvinylpyrrolidone (PVP) as an agglutination-augmenting vehicle. PVP K 60 (MW 160,000) was obtained as a 45% aqueous solution. The tiration medium (PVP-BSA) was prepared by dissolving 300 mg of PVP solution into 10 ml of 5% W/V solution of bovine serum albumin (BSA) in phosphate buffered saline (PBS). The PVP-BSA was dispensed in 50 µl

aliquots into rows of 6 X 50 mm glass tubes. Aliquots of 50 µl of antisera were placed in the first tube of each row and serial dilutions were made to a final $\log_2$ of 15. A 3% (V/V) target RBC suspension was dispensed in 50 µl aliquots to each tube containing diluted antiserum as well as control tubes. After gentle mixing the tubes were incubated for 1 hour at 37°C followed by 2 hours at 4°C. Following incubation the contents of each tube were gently aspirated with a Pasteur pipette and streaked on a glass plate. The plate was rocked gently to disperse the RBC in the streaks, and hemagglutination was scored both macroscopically and microscopically on a scale from 1 to 5 on the basis of increasing agglutination. The titre end point was taken as the last dilution at which the hemagglutination score was greater than 1 point above that for normal mouse serum.

Statistical methods.

Median survival times (MST) and their 95% confidence limits (CL) and P values were calculated using the nomograph method of Litchfield (7). Means ($\bar{X}$) and standard errors (SE) for hemagglutination end points and PFC responses were calculated by the least squares method (9) on a Hewlett-Packard calculator, model 9100A.

Experimental Protocol.

The methods described above were employed during the performance of the experiments discussed in this text. For clarity, the protocol

of each experiment is outlined in the following chapters just prior to the description of experimental observations.

CHAPTER 2: REFERENCES

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CHAPTER 3: RESULTS

Factors Determining the Extent of Immunosuppression

Effect of Strength and Duration

The degree of immunosuppression brought on by a GVH reaction has been shown to depend upon two primary factors. The first factor is the strength of the GVH reaction. Strength, in turn, is determined by histocompatibility differences between donor and host, and by the source and dose of donor cells. The second factor, which is equally as important, is the duration of the GVH reaction at the time of immunization with the test antigen. The importance of these 2 factors in determining the immune responsiveness of the host can be illustrated by 2 simple experiments.

GVH reactions were initiated in 2 F_1 hybrid combinations selected to produce reactions of different intensity. A moderate GVH was induced by the intravenous injection of 75 x 10^6 A spleen and lymph node cells into adult CBA x A F_1 (CAF_1) mice. The H-2^a (A strain) is thought to be derived from recombination between H-2^k and H-2^d and therefore shares the same K region as H-2^k (CBA) (7). As a result, GVH reactions in this strain combination were of a moderate intensity with an 85% survival rate and a median survival time of greater than 100 days (Table 1). GVH reactions of a more severe nature were induced by a similar injection of A strain lymphoid cells into adult

C57BL/6 x A F_1 ($B6AF_1$) mice. The H-2 genotypes in these strains ($H\text{-}2^b$ and $H\text{-}2^a$ respectively) are of independent origin and have a genetic disparity which caused 100% mortality and a median survival time of 35 days (Table 1). Experimental animals and normal littermate controls for both F_1 combinations were then separated into groups and each group was sensitized to SRBC at a different time post GVH. Four days after sensitization to SRBC, PFC assays were performed to determine the humoral response to SRBC.

The variability of the conditions of sensitization to SRBC and assay is reflected in the wide range of PFC responses seen in normal animals treated on different days (Table 2). Animals treated at the same time, however, show much more consistent responses. For this reason the PFC response of GVH mice is expressed as the per cent of that observed in normal controls sensitized and assayed at the same time.

When sensitized to SRBC 1 day after GVH induction, CAF_1 mice exhibited a PFC response 4 days later which was 86.9% of that seen in normal, sensitized controls (Table 2). In this CAF_1 strain combination, increases in the PFC response were observed when GVH animals were sensitized to SRBC 2 days and 3 days after the mice received parental cells. The PFC response rose 5.9% and 45.3% above normal when GVH mice were sensitized to SRBC on days 2 and 3 respectively. When sensitized on day 5 post GVH induction, however, the PFC response fell to only 15.9% of normal, and when sensitized on day 7 the response in

TABLE 1

Survival Time[a] (Days) of CAF_1 and $B6AF_1$ Mice Receiving 75×10^6 A Lymphoid Cells Intravenously.

	CAF_1	$B6AF_1$
Mouse Survival Time (Days)	22 62 >75 >75 >75 >100 >100 >100 >100 >100 >100 >100	15 15 16 19 41 44 47 47 48 70
	MST>100	MST 35 (32.1 - 38.2)

[a] Median survival times and their 95% confidence limits were determined using the nomograph method of Litchfield (10).

TABLE 2

PFC Response of CAF_1 Mice Sensitized to SRBC

at Various Intervals After GVH Induction.

Day Post GVH When Sensitized to SRBC	GVH Mice PFC per spleen ($\times 10^3$)	Normal Mice PFC per spleen ($\times 10^3$)	GVH % of Normal Response
Day 1	44.7 70.8 87.3 116.7 $\bar{X} = 79.9 \pm 15.1$	50.1 85.8 96.0 135.6 $\bar{X} = 91.9 \pm 17.6$	86.9
Day 2	271.8 324.2 336.0 364.8 $\bar{X} = 324.2 \pm 19.4$	278.4 301.2 310.2 334.2 $\bar{X} = 306.0 \pm 11.5$	105.9
Day 3	236.7 243.3 261.6 292.8 $\bar{X} = 258.6 \pm 12.6$	111.9 119.1 196.2 284.4 $\bar{X} = 178.0 \pm 40.3$	145.3
Day 5	18.0 18.3 25.8 27.6 $\bar{X} = 22.4 \pm 2.5$	135.5 136.8 144.2 146.3 $\bar{X} = 140.7 \pm 2.7$	15.9
Day 7	0.6 0.9 1.5 2.7 $\bar{X} = 1.4 \pm 0.5$	153.9 237.9 240.0 302.1 $\bar{X} = 233.5 \pm 30.4$	0.6

(Continued).....

TABLE 2 (Continued)

Day Post GVH When Sensitized to SRBC	GVH Mice PFC per spleen ($\times 10^3$)	Normal Mice PFC per spleen ($\times 10^3$)	GVH % of Normal Response
Day 70	0.0 0.0 0.0 0.0 0.0 $\bar{X} = 0.0 \pm 0.0$	155.7 176.1 212.1 213.0 344.4 $\bar{X} = 220.3 \pm 32.9$	0.0
Day 100	0.0 0.0 0.0 0.3 0.3 0.6 $\bar{X} = 0.2 \pm 0.1$	121.5 174.6 278.7 $\bar{X} = 191.6 \pm 46.1$	0.0

[a] Mean values $\pm$ SE are given following individual responses for each group.

GVH mice was no greater than that of normal mice not sensitized to SRBC. Although these animals frequently appeared to recover later from their GVH reactions, the PFC response to SRBC remained completely suppressed in these animals through day 100 post GVH. This finding of an initial increased responsiveness after GVH induction followed by complete suppression has also been reported in the same strain combination by Davis et al. (2).

In more severe GVH reactions the initial increase in responsiveness is much less significant and immunosuppression appears much sooner than with a moderate GVH reaction. This can be seen from Figure 1 (8) which represents the response to SRBC in $B6AF_1$ mice injected intravenously with 75 x 10^6 A spleen and lymph node cells. The injection of SRBC immediately following parental cells produced a PFC response 4 days later that was 23% greater than that for normal controls receiving SRBC. The onset of immunosuppression was much more rapid in $B6AF_1$ mice, and by the second day post GVH stimulation with SRBC produced a PFC response which was 46% that in normal animals. The reactivity of GVH animals fell to 16% of normal on day 4 and suppression was complete within 7 days after GVH induction.

The effect of GVH strength and duration on immune responsiveness is summarized in Figure 1. The increased responsiveness observed at day 3 in CAF_1 GVH mice closely resembles the allogeneic effect described by Katz (5,6,11) with the exception that in the present experiment the mice were not sensitized to SRBC prior to receiving parental cells.

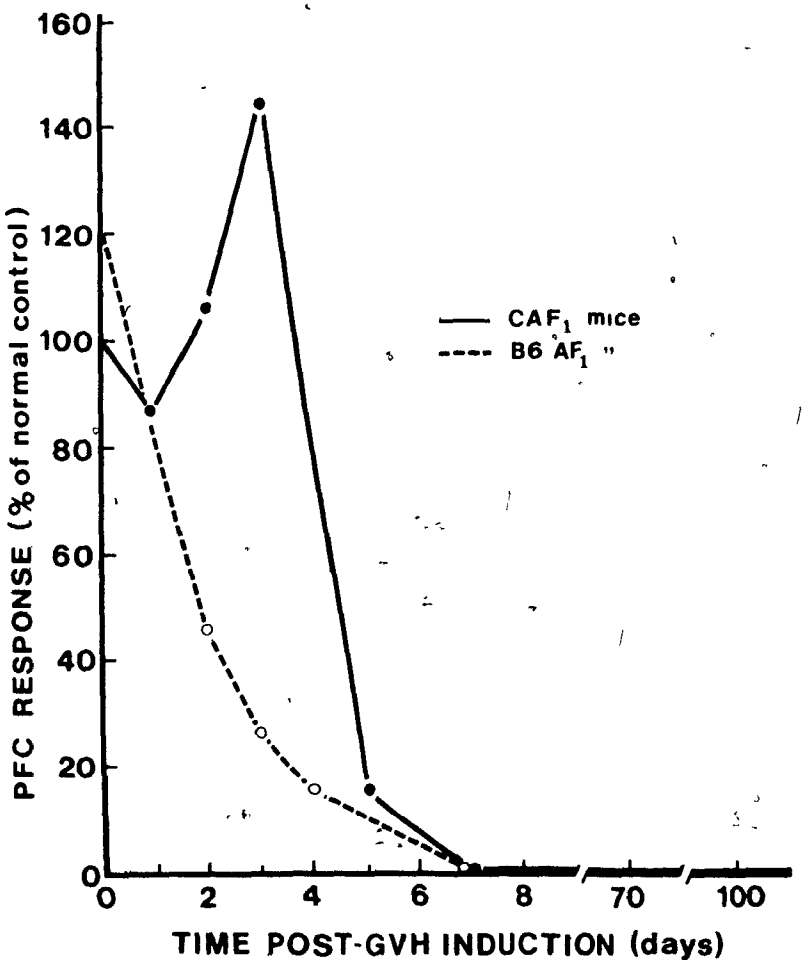


FIGURE 1: PFC Response of CAF_1 and $B6AF_1$ Mice Sensitized to SRBC at Different Times Following GVH induction.

It is doubtful that the increased responsiveness in CAF_1 mice results merely from the increased number of immunocompetent cells in the F_1 host, since this effect was not observed until 3 days after GVH induction. The 3 day time lag suggests that proliferation of the donor cells following stimulation by F_1 alloantigens is necessary for the appearance of increased responsiveness. Furthermore, this effect appeared to be non-specific since stimulation by alloantigens resulted in a heightened response to an unrelated antigen. Both these findings are consistent with the Katz proposal that the allogeneic effect results from the production by GVH reactive cells of non-specific T cell secreted mediators with the capacity to stimulate B cells to antibody formation following their interaction with antigen. The subsequent decrease of immunologic responsiveness which was observed as the GVH reaction progressed has also been reported for the allogeneic effect (11), and might easily result from increased utilization and depletion of such thymic mediators by the progressing GVH reaction. This possibility will be dealt with in more detail later.

In the course of the GVH reaction in $B6AF_1$ mice, a more rapid onset of immunosuppression appeared. An increased response was observed when mice were sensitized to SRBC immediately after receiving parental cells, but it was difficult to determine whether this increase resulted from an allogeneic effect. The immediate nature of the effect, however, suggested that the effect resulted from the larger number of immunocompetent cells in the $B6AF_1$ host. Since GVH reactions are

very strong in this strain combination, it seemed likely that the rapid onset of immunosuppression arose from a stronger immunologic attack by parental cells on the F_1 host. This could result in a rapid destruction of host lymphoid cells, or in a rapid depletion of thymic mediators by cells engaged in the vigorous GVH reaction. Both of these results could contribute to immunosuppression.

It is well established in a wide variety of animals that the degree of immunosuppression increases with the strength of the GVH reaction. The strong immunologic attack upon an animal undergoing a GVH reaction, however, represents a profound physiological insult and results in considerable stress to the host. Since stress also increases with the intensity of the GVH reaction, it is possible that GVH induced immunosuppression results from a general stress response rather than from a definite immunological consequence of the GVH reaction. The next series of experiments was performed to determine whether stress was responsible for the immunosuppression observed in animals undergoing GVH reactions.

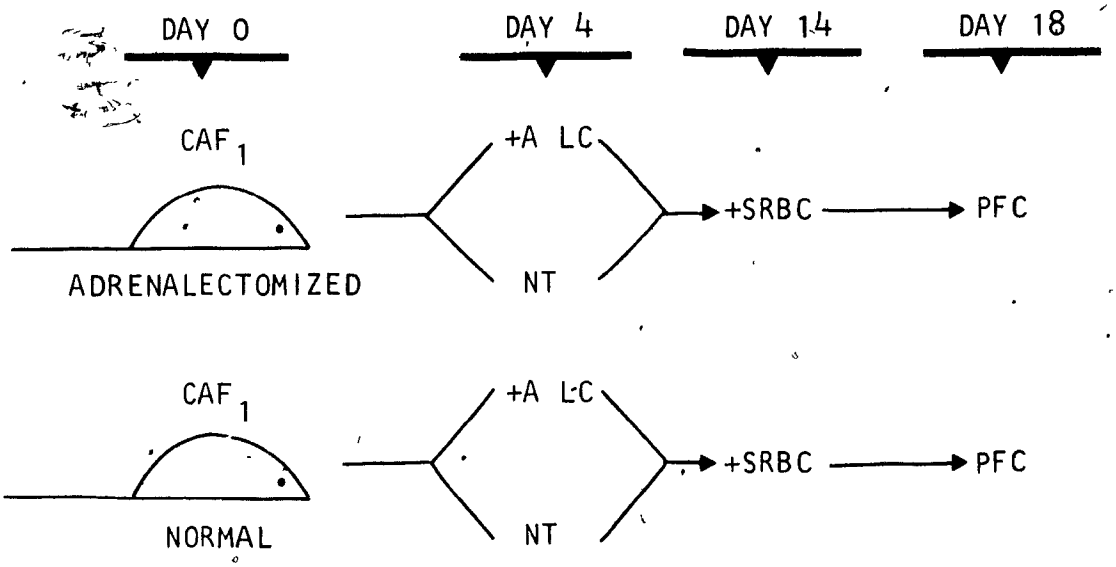
Effect of Adrenalectomy on the Response of GVH Mice to SRBC.

Numerous reports (1,3,9) have shown that hormones associated with the adrenal glands can suppress immunological activity. Hydrocortisone, for example, has been shown to suppress proliferative and antibody forming responses of spleen cells in vivo (9), while the ability to initiate a GVH reaction remains intact (1). It was considered possible that a

host response to the stress produced by a GVH reaction could cause the release of sufficient hormones from the adrenal glands to bring about a generalized suppression of the host's immune system. The following experiment was therefore carried out to determine if GVH immunosuppression could be explained in terms of a general stress response or whether it was a direct immunological consequence of donor and host cell interactions during the GVH reaction.

Bilateral adrenalectomies were performed on adult CAF_1 male mice while a group of littermates received sham operations. Four days after adrenalectomy, GVH reactions were induced in one half of these mice in the manner previously described (Figure 2). All mice were sensitized to SRBC 10 days later and the number of splenic PFC to SRBC was determined 4 days after sensitization. Using the same protocol, mice with intact adrenal glands were given GVH reactions, sensitized to SRBC and tested for PFC. Throughout the experiment all animals were allowed a choice of tap water or a 0.5% solution of NaCl ad libitum.

Four days after being sensitized to SRBC, normal animals with intact adrenal glands showed a mean direct PFC response of 158.2×10^3 (Table 3 and Figure 3 represent data pooled from 3 experiments). There was no significant difference between the PFC response in non-treated (non-adrenalectomized) animals and that of littermates receiving sham operations. In contrast, adrenalectomized littermates exhibited a mean direct PFC response of 251.8×10^3, representing a 60% increase over the response in normal animals. These findings



A LC - A Strain Lymphoid Cells; NT - No Treatment;

FIGURE 2: Design of Experiment to Determine the Effect of Adrenalectomy on the Response of GVH Mice to SRBC.

TABLE 3

Direct PFC Response to SRBC in Normal and Adrenalectomized Mice Undergoing GVH Reactions[a,b]

Experimental Group	Normal PFC per spleen x 10^3	GVH PFC per spleen x 10^3
Adrenals Intact	59.7 117.9 139.8 149.1 150.3 156.0 166.2 172.5 183.3 193.2 196.5 213.9 $\bar{X}$ = 158.2 ± 11.8	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.3 0.3 0.3 0.3 1.2 1.2 1.2 3.6 4.2 4.8 $\bar{X}$ = 0.9 ± 0.3
Shams	67.2 [illegible]5.9 107.4 110.7 115.5 121.8 125.4 138.9 167.1 171.3 175.5 177.3 217.8 251.4 $\bar{X}$ = 146.7 ± 13.2	0.0 0.0 0.0 0.0 0.0 0.3 0.3 0.6 0.6 0.6 2.1 25.2 $\bar{X}$ = 2.5 ± 2.1
Adrenalectomized	146.7 166.2 170.7 180.6 204.9 207.6 220.2 231.6 234.9 237.3 259.8 281.1 300.6 305.1 313.8 320.7 322.5 326.4 353.7 $\bar{X}$ = 251.8 ± 14.5	0.0 0.0 0.0 0.3 0.3 0.3 0.3 0.6 0.6 1.2 1.8 5.7 8.1 8.1 15.6 $\bar{X}$ = 2.9 ± 1.2

[a] Data represents data pooled from 3 experiments.

[b] Mean values ± SE are given following individual responses for each group.

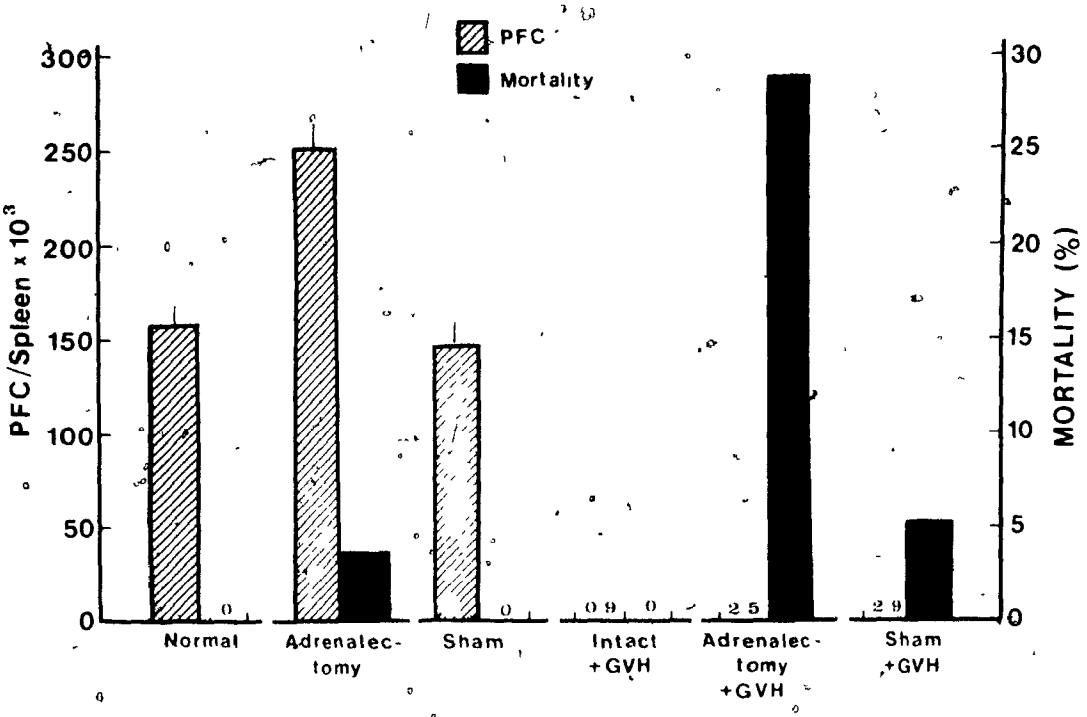


FIGURE 3: Direct PFC Response to SRBC and Mortality Rate in Normal and GVH Mice Following Adrenalectomy.

would suggest that hormones from the adrenal glands play a suppressive role in normal animals and control the extent to which the animal can respond to certain antigens. Animals with all glands intact but experiencing GVH reactions showed no PFC response to SRBC.

A very slight PFC response was seen in groups of GVH animals which had been adrenalectomized, but this value was almost identical to that for the corresponding groups of GVH mice receiving sham operations.

It has been well established that sympathetic stimulation of the adrenal glands during stress causes them to release hormones which play an important role in the animal's response to the stress. A GVH reaction undoubtedly represents a considerable stress to the host, and this was evident from the incidence of mortality which was higher in adrenalectomized-GVH mice than in mice with either adrenalectomy alone or GVH alone (Table 4 and Figure 3). Of 28 mice in each experimental group, adrenalectomized mice undergoing GVH reactions had a mortality rate of 28.6%, while those receiving only adrenalectomies had a rate of 3.6% and those given sham operations and GVH reactions had a mortality rate of 5.2%. The group of intact mice experiencing GVH reactions showed no deaths. Chi square analysis of mortality rates showed a significant difference ($P < 0.01$) between GVH mice which had been adrenalectomized and GVH mice receiving sham operations. From these experiments it is difficult to pinpoint the precise cause of the increased mortality observed in GVH mice which had been previously adrenalectomized. The in-

TABLE 4

Mortality Rate of Adrenalectomized Animals

Undergoing GVH Reactions[a].

	Individual Mortality	Total Mortality	Per Cent Mortality
Normal	0/8 0/8 0/6 0/6	0/28	0
Adrenalectomy	0/8 0/8 0/6 0/6	1/28	3.6
Sham	0/5 0/5 0/5 0/4	0/19	0
Adrenals Intact & GVH	0/8 0/8 0/6 0/6	0/28	0
Adrenalectomy & GVH	4/8 0/8 1/6 3/6	8/28	28.6
Sham & GVH	0/5 0/5 0/5 1/4	1/19	5.2

[a] Data indicates number of deaths over number of experimental animals in four separate experiments.

creased death may result from a decreased capacity of these animals to adapt to generalized stress. Previous data, however, (Table 3) has suggested that the adrenal glands may help control the extent of normal immune responses. It is also possible therefore, that adrenal hormones may influence the intensity of GVH reactions, and that adrenalectomy may remove some of this control, leading to more intense GVH activity and increased mortality.

Although the mortality rate data (Table 4) suggest that adrenal hormones may lessen the overall intensity of the GVH reaction, it would appear that they do not play a significant role in the immunosuppressive effect of the GVH reaction. The fact that immunosuppression was as complete in GVH animals lacking adrenals as it was in those with intact glands strongly suggests that this suppression is not brought about simply by hormones released from the adrenals in response to stress. Rather, it suggests that GVH induced immunosuppression results from some other immunological consequence of the GVH reaction.

It is interesting to note that Graff et al. (4) have shown that steroids from the gonads as well as those from the adrenals may have a moderating influence on immune responses. Castration or adrenalectomy of male mice caused an accelerated rejection of subsequent skin grafts differing at a minor H locus. Combined adrenalectomy and castration caused an even faster rejection of such skin grafts, although the difference between the combined removal and either castration or adrenalectomy alone was not statistically significant. In the present series

of experiments, an experiment was performed in which one group of mice was both castrated and adrenalectomized, after which one half were subjected to a GVH reaction. The data from this experiment appear in Table 5. Following sensitization with SRBC, mice receiving adrenalectomy and castration but no GVH exhibited a mean response of 312.0 x 10^3 PFC per spleen. This response compared to mean values of 227.0 x 10^3 PFC in adrenalectomized mice and 162.6 x 10^3 PFC in normal animals. These results are similar to those reported by Graff et al. and suggest that steroids from the gonads as well as from the adrenal glands may exert a moderating effect on the immune responsiveness of normal mice. The removal of both the adrenal glands and the testes, however, failed to restore the response of GVH mice to SRBC, and no PFC could be detected in these mice. This finding lends further support to the contention that GVH induced immunosuppression is produced by definite immunological causes rather than by the release of steroid hormones in response to stress.

TABLE 5

Effect of Adrenalectomy and Castration on the PFC Response to SRBC in Normal and GVH Mice[a].

Experimental Group	Normal PFC per spleen x 10^3	GVH PFC per spleen x 10^3
Adrenals Intact	117.9 156.0 183.3 193.2 $\bar{X}$ = 162.6 ± 16.9	0.0 0.0 0.0 0.0 0.3 1.2 $\bar{X}$ = 0.3 ± 0.2
Sham	107.4 115.5 171.3 177.3 $\bar{X}$ = 142.9 ± 18.3	0.0 0.0 0.3 0.3 $\bar{X}$ = 0.2 ± 0.2
Adrenalectomized	166.2 207.6 220.2 259.8 281.1 $\bar{X}$ = 227.0 ± 20.2	0.0 0.0 0.3 0.3 $\bar{X}$ = 0.2 ± 0.1
Adrenalectomized and Castrated	224.1 224.4 253.2 324.9 390.0 455.1 $\bar{X}$ = 312.0 ± 39.0	0.0 0.0 0.3 0.3 0.6 $\bar{X}$ = 0.2 ± 0.1

[a] Mean values ± SE are given following individual responses for each group.

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CHAPTER 4: RESULTS

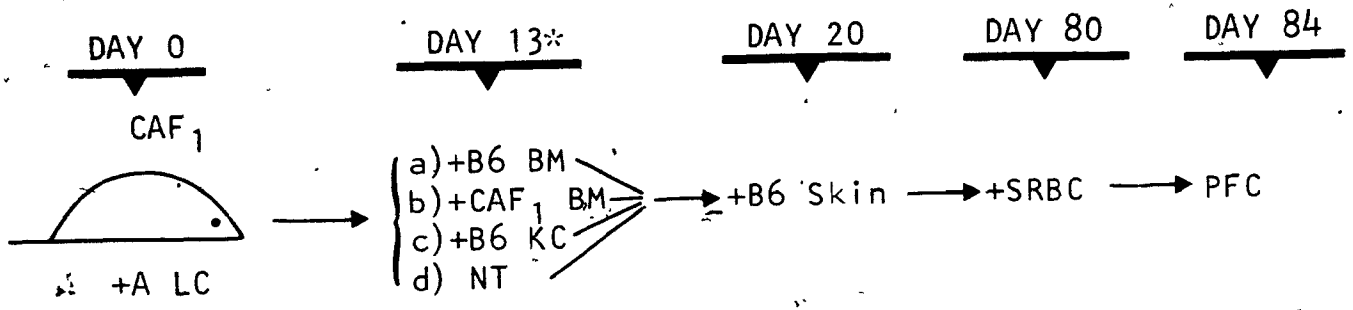
Effect of Multiple Antigenic Challenges on Cell-Mediated and Humoral Immune Responses.

Introduction

The previous experiments established that the immunosuppression observed during a GVH reaction apparently results from immunologic causes. In all studies thus far dealing with GVH induced immunosuppression, however, the animals have been challenged only once following the induction of the GVH reaction. In addition, no attempts have been made to determine the effects of the GVH reaction on the humoral versus the cell-mediated immune response under conditions of multiple antigenic stimulation. Elucidation of these effects could provide further insight into the underlying causes of immunosuppression. The following studies were therefore carried out to determine the effect of multiple antigenic challenges on both humoral and cell-mediated immune responses in animals experiencing GVH reactions (11,12).

The Effect of Antigenic Stimulation on Skin Allograft Survival Time.

To test the effect of antigenic stimulation on skin allograft survival time, GVH reactions were initiated in adult CAF_1 male mice by the intravenous injection of 75×10^6 A strain spleen and lymph node cells (Figure 4). Thirteen days later the animals were divided into four



*) Following antigenic challenge, 4 mice from each experimental group were sensitized to SRBC on day 13 and assayed for splenic PFC on day 17.

A LC - A Strain Lymphoid Cells; BM - Bone Marrow;
KC - Kidney Cells; NT - No Treatment;

FIGURE 4: Design of Experiment to Determine the Effect of Antigenic Stimulation on Skin Allograft Survival Time in GVH Mice.

groups, each receiving one of the following treatments: 40 x 10^6 B6 BM cells, B6 kidney cells, 40 x 10^6 CAF_1 BM cells, or no further treatment. On day 20 post-GVH the remaining mice were grafted with skin allografts from B6 mice. Two such experiments were carried out and the results of each were pooled.

First-set responses to B6 skin grafts made 20 days after initiation of the GVH reaction were variable, depending on the treatment which the animals received prior to skin grafting (Table 6). The MST of B6 skin on CAF_1 GVH mice was 21.5 days, contrasting to a MST of 7.9 days for normal animals in this strain combination. B6 skin grafts had a MST of only 8.1 days in the group which received a GVH plus B6 BM, while those that received B6 kidney cells had a longer allograft MST of 11.0 days. The longest allograft survival time was 26.0 days, seen in the group of GVH mice receiving F_1 BM. The difference between the MST for GVH mice receiving F_1 BM and the MST for the group which received a GVH alone was not statistically significant ($P > 0.05$).

When second-set B6 skin grafts were applied following first-set rejection, the survival time of the second-set grafts was shorter than that for the first-set in all groups except the recipients of B6 kidney cells (Table 6). In the group receiving only a GVH the MST decreased from 21.5 days to 8.2 days; and in the group receiving F_1 BM it decreased from 26.0 to 13.0 days. Although the survival time of second-set grafts in the animals receiving B6 kidney cells appeared slightly longer than that for the first-set, the difference between the two values was not signi-

TABLE 6

Survival Times of First and Second-Set B6 Skin Allografts

On CAF_1 GVH Mice Stimulated with B6 BM

B6 Kidney Cells, or F_1 BM.

Treatment	First-Set Graft Survival[a]	Second-Set Graft Survival[a]
Normal F_1	7 7 7 8 8 8 9 MST = 7.9 (7.7 - 8.1)	6 6 6 7 7 7 8 MST = 6.9 (6.7 - 7.1)
GVH Alone[b]	15 16 17 18 18 21 21 22 24 25 28 28 30 49 92 MST = 21.5 (18.2 - 25.4)	6 7 8 9 10 12 MST = 8.2 (6.8 - 9.9)
GVH + B6 BM	7 7 7 7 7 8 8 8 9 10 10 10 11 15 15 MST = 8.1 (7.1 - 9.2)	6 6 6 7 9 20 MST = 6.5 (5.0 - 8.4)
GVH + B6 Kidney Cells[b]	10 10 10 11 11 11 11 14 14 28 30 MST = 11.0 (9.1 - 13.9)	9 10 13 15 20 40 MST = 13.0 (9.6 - 17.5)

(Continued).....

TABLE 6 Continued.

Treatment	First-Set Graft Survival[a]	Second-Set Graft Survival[a]
GVH+F_1 BM[c]	11 17 22 23 25 25 36 43 56 72	7 8 13 20 29
	MST = 26.0 (17.7 - 38.0)	MST = 13.0 (7.9 - 21.4)

[a]median survival time (MST) and 95% confidence limits (CL) were determined by the method of Litchfield (7).
[b]the MST of grafts on GVH Mice was not significantly different from the MST of grafts on GVH mice treated with F_1 BM ($P > 0.05$). Likewise the difference between the second-set MST for these two groups was also not significant ($P > 0.05$).
[c]the MST for second-set grafts in this group was not significantly different from the first-set MST ($P > 0.05$).

ficant ($P > 0.05$).

It is important to know the extent to which the animals were immunosuppressed at the time of stimulation with allogeneic cells. Lawrence and Simonsen (6) have shown a correlation between the strength of a GVH reaction and its ability to suppress the PFC responses to SRBC. To test for immunosuppression by the GVH reaction, four mice from each experimental group outlined above were selected at random and sensitized to SRBC on day 13 post GVH, 4 hr after the injection of BM or kidney cells. The number of splenic PFC in these mice was determined 4 days later (Table 7). Animals which received a GVH alone as well as GVH mice which received BM or kidney cells had mean values of less than 0.3×10^3 PFC per spleen. This was in contrast to normal animals which had a mean PFC response to SRBC of 121.0×10^3 PFC per spleen.

At the termination of the experiment, 80 days post GVH all animals which had rejected B6 skin grafts were sensitized to SRBC, and the number of PFC was determined 4 days later. Table 7 indicates that the PFC response to SRBC was almost completely suppressed in each experimental group. The highest mean PFC count was 0.9×10^3 PFC per spleen in the group which received F_1 BM. The values in each group were comparable to background levels seen in unsensitized animals and were negligible when compared to the mean value of 213.9×10^3 PFC per spleen in sensitized littermate controls not experiencing a GVH reaction.

These results demonstrate that both the cell-mediated and humoral immune responses are suppressed during an ongoing GVH reaction. Further-

TABLE 7

Direct PFC to SRBC in GVH Mice After Stimulation with Allogeneic Cells and Rejection[a] of Allogeneic Skin Grafts.

Treatment	Day 13[b] PFC per spleen X 10^3	Day 80[c] PFC per spleen X 10^3
GVH	0.0 0.0 0.0 0.0 0.0 0.0 $\bar{X}$ = 0.0	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.3 0.6 1.8 $\bar{X}$ = 0.2 ± 0.1
GVH + B6 BM	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 $\bar{X}$ = 0.0	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.3 0.3 0.6 0.9 1.2 3.6 5.1 $\bar{X}$ = 0.8 ± 0.4
GVH + B6 Kidney Cells	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 $\bar{X}$ = 0.0	0.0 0.0 0.0 0.0 0.3 0.6 0.6 0.6 0.9 $\bar{X}$ = 0.3 ± 0.1
GVH + F_1 BM	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 $\bar{X}$ = 0.0	0.0 0.0 0.0 0.3 0.6 1.2 1.2 2.1 2.4 $\bar{X}$ = 0.9 ± 0.3

(Continued).....

TABLE 7 Continued.

Treatment	Day 13[b] PFC per spleen X 10^3	Day 80[c] PFC per spleen X 10^3
Normal	43.8 59.1 67.5 78.6 134.7 175.5 289.5	155.7 176.1 193.5 204.9 208.2 212.1 213.0 221.7 344.4
	$\bar{X} = 121.0 \pm 33.0$	$\bar{X} = 213.9 \pm 18.0$

[a]mean values ± SE are given following individual responses for each group.
[b]randomly selected animals from each experimental group were injected with SRBC on day 13, 4 hr after the injection of allogeneic cell suspensions.
[c]experimental mice described in Table 1 were sensitized to SRBC following skin graft rejection at day 80 and analyzed for splenic PFC 4 days later.

more, the results show that the cell-mediated response can be stimulated in GVH mice by repeated sensitization with allotransplantation antigens. The results also show that the humoral immune response to SRBC cannot be stimulated by similar repeated challenges with SRBC. This would suggest that GVH-induced immunosuppression has a greater effect on the humoral immune response than on the cell-mediated response. From the data presented here, however, a basic uncertainty remains. The previous results show that it is possible to stimulate a cell-mediated immune reaction to transplantation antigens in GVH animals while the same animals fail to produce a detectable direct PFC response to SRBC. Two important questions remain. Since the direct PFC assay measures only the production of IgM antibody, it is impossible to state whether these animals are capable of mounting an IgG response to SRBC. Secondly, one might ask whether it was possible to detect any humoral response to the same H antigens which stimulated graft rejection.

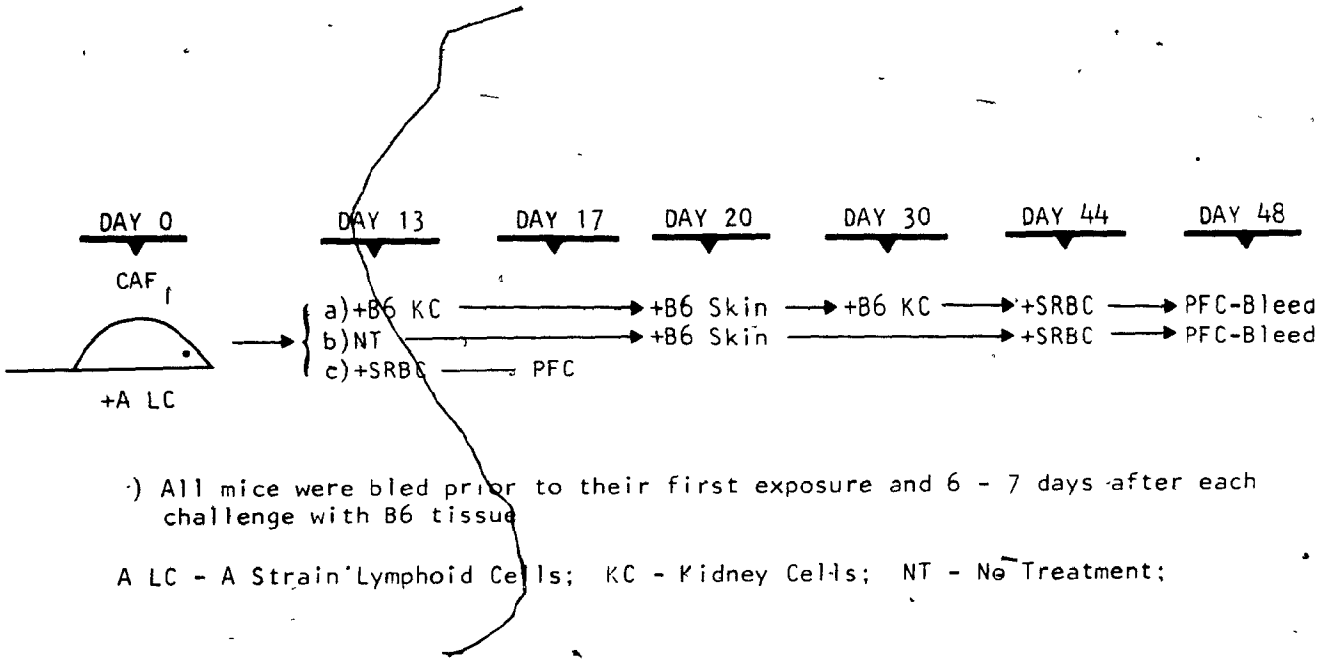
Antibody Activity in GVH Immunosuppressed Mice Stimulated with Transplantation Antigen.

A possibility exists that H antigens may provide a stronger antigenic stimulus than SRBC, and with appropriate stimulation may be capable of eliciting a humoral immune response in GVH immunosuppressed animals. The following study was therefore undertaken to determine if a humoral immune response to H antigens could be detected in GVH immunosuppressed mice in which cell-mediated reactions had been stimu-

lated to the same H antigens (13).

GVH reactions were induced by the intravenous injection of 75×10^6 living A strain spleen and lymph node cells into adult CAF_1 male mice (Figure 5). Thirteen days post GVH the animals were given intraperitoneal injections of B6 kidney suspensions, followed on day 20 with skin allografts from B6 mice, and another injection of B6 kidney on day 30. Seven days after each exposure to B6 antigen, mice were randomly selected from each experimental group, bled through the retro-orbital sinus and their sera collected and stored at -30°C. The amount of circulating antibody in their sera to B6 alloantigens was determined by the agglutination of B6 red blood cells using polyvinylpyrrolidone (PVP) as an agglutination augmenting vehicle. To test for the humoral response to SRBC, randomly selected mice from each experimental group received intravenous injections of 5×10^8 SRBC on day 13 post GVH, and 4 days later the number of PFC in their spleens was determined. Similarly, 14 days after their last exposure to B6 antigen, all mice were sensitized to SRBC and the number of splenic PFC determined 4 days later.

B6 skin grafts made on CAF_1 mice 20 days after the initiation of a GVH reaction had an MST of 20.0 days (Table 8). This contrasted to an MST of 8.0 days in normal mice of the same strain combination. Sensitization of GVH mice with B6 kidney cells 7 days prior to skin grafting reduced the MST to 11.5 days, contrasting to an MST of less than 7 days for normal CAF_1 mice which had been previously sensitized with B6 kidney cells. The differences in MST between each experimental group are



·) All mice were bled prior to their first exposure and 6 - 7 days after each challenge with B6 tissue

A LC - A Strain Lymphoid Cells; KC - Kidney Cells; NT - No Treatment;

FIGURE 5: Design of Experiment to Determine the Effect of Antigenic Stimulation on the Humoral Immune Response to Transplantation Antigens in GVH Mice.

TABLE 8

Survival Time of B6 Skin Allografts on CAF_1 GVH Mice Treated with B6 Kidney Cells.

Treatment	Graft Survival Time (Days)	MST[a]	95% CL[a]
Normal	7 7 7 7 7 8 8 8 8 8 8 8 8 9 9 9 9 9 9	8.0	7.3 - 8.8
Normal + B6 Kidney Cells	< 7 < 7 < 7 < 7 < 7 < 7	< 7	
GVH Alone	10 11 14 15 15 16 16 17 17 18 18 20 21 21 22 22 24 24 25 28 28 30 51 92	20.0	17.2 - 23.2
GVH + B6 Kidney Cells	7 7 9 10 10 10 11 11 11 11 11 11 12 13 13 14 14 16 17 28 >30	11.5	9.8 - 13.5

[a]median survival time (MST) and 95% confidence limits (CL) were determined by the method of Litchfield (7).

statistically significant ($P<0.05$) and are comparable to the values previously reported in the same strain combinations.

Both normal and GVH mice had no detectable hemagglutinating antibodies to B6RBC prior to being sensitized with B6 tissue (Table 9). Six days after receiving injections of B6 kidney suspension, normal CAF_1 mice showed a mean $\log_2$ hemagglutinating titre to B6RBC of 7.50 while GVH recipients of B6 kidney had no detectable B6 hemagglutinating antibodies. In the same mice 7 days after a subsequent B6 skin graft, the normal group had a mean $\log_2$ hemagglutinin titre to B6RBC of 5.20 while the GVH group still showed no detectable humoral response to B6 antigen. Six days following the second injection of B6 kidney the mean $\log_2$ hemagglutinating titre for normal mice had risen to 9.17 while there remained no detectable response in GVH mice receiving the same treatment.

These results once again showed that repeated sensitization of GVH mice with B6 allotransplantation antigen stimulated a cell-mediated response which resulted in a rapid rejection of B6 skin grafts. At no time, however,was there any detectable humoral response in these mice to the same B6 antigens.

It has been reported by Stimpfling (10) that red blood cells from C57BL mice tend to exhibit weaker agglutination reactions with specific antisera than do red cells of many other strains. The use of PVP, however, as a developing agent in mouse hemagglutination tests has been shown to provide a sensitive test for low levels of specific hemagglutinating antibodies (3,10). In the experiments represented in Table 9 normal mice developed a significant level of antibody against B6 antigens

TABLE 9

Hemagglutinating Antibody Titre (log_2) to B6 Alloantigens in CAF_1 Normal and GVH Mice Treated with B6 Kidney Cells and Skin Grafts ($\pm$ SE)[a].

Treatment	Days After First B6 Challenge	Normal Antibody Titres[b]	GVH Antibody Titres
No Treatment		0 0 0 0 0 0 0 0 0 0 $\bar{X} = 0 \pm 0$	0 0 0 0 0 0 0 0 0 0 $\bar{X} = 0 \pm 0$
B6 Kidney	6	7 7 7 7 8 9 $\bar{X} = 7.50 \pm 0.34$	0 0 0 0 0 0 $\bar{X} = 0 \pm 0$
B6 Kidney + B6 Skin	14	0 0 0 5 5 6 6 10 10 10 $\bar{X} = 5.20 \pm 1.30$	0 0 0 0 0 0 0 0 0 0 $\bar{X} = 0 \pm 0$
B6 Kidney + B6 Skin + B6 Kidney	23	7 8 10 10 10 10 $\bar{X} = 9.17 \pm 0.54$	0 0 0 0 0 0 0 0 0 0 $\bar{X} = 0 \pm 0$

[a]mean values $\pm$ SE are given following individual responses for each group.
[b]all animals were bled 6 to 7 days after challenge with B6 kidney cells and/or B6 skin grafts.

within 6 days after their first exposure to B6 antigens. The slight decrease in the level of circulating antibody following grafting with B6 skin might be accounted for by the absorption of antibody to the graft. After 3 exposures to B6 antigens, normal CAF_1 mice exhibited a $\log_2$ hemagglutinin titre of 9.25, a level comparable to that reported by Stimpfling for the same H-2 specificities.

Response of GVH Mice to SRBC Before and After Exposure to B6 Antigen.

A PFC assay was performed on randomly selected mice to test the ability of normal and GVH mice to mount a humoral response to SRBC both before and after challenge with B6 antigen. GVH animals which were sensitized to SRBC on day 13 post GVH showed no direct PFC response to SRBC when assayed 4 days later (Table 10). Normal animals injected at the same time with SRBC had a mean value of 441.2×10^3 PFC per spleen. Similarly, when sensitized to SRBC after 3 exposures to B6 tissue, GVH mice showed no direct PFC response to SRBC 4 days later. Normal mice receiving the same treatment with B6 antigen followed by SRBC had a mean count of 237.6×10^3 PFC per spleen. The PFC response in these mice was almost identical to that observed in normal mice receiving only one exposure to B6 antigen.

The absence of any detectable level of anti-B6 antibody in GVH mice receiving 3 challenges with B6 tissue strongly suggests that the humoral response to B6 antigens remains completely suppressed in these animals. The absence of any PFC response to SRBC in these GVH mice lends further support to the contention that the overall humoral response remains com-

TABLE 10

Direct PFC to SRBC in GVH Mice Prior to and Following Stimulation with B6 Kidney Cells and Rejection of B6 Skin Grafts[a].

Treatment	PFC/Spleen X 10^3 ± SE at days 13 and 44 post-GVH	
	Day 13	Day 44
Normal	374.7 421.5 460.5 466.2 483.3 $\bar{X}$ = 441.2 ± 19.5	163.5 167.4 168.9 169.2 189.0 200.1 225.0 240.6 241.5 267.3 323.1 406.8 $\bar{X}$ = 230.2 ± 21.3
Normal + B6 Kidney 2X	ND[c]	71.1 187.2 188.1 193.8 223.2 237.9 253.2 306.0 332.4 383.4 $\bar{X}$ = 237.6 ± 27.9
GVH	0.0 0.0 0.0 0.0 0.3 0.3 $\bar{X}$ = 0.1 ± 0.1	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.3 0.3 0.3 $\bar{X}$ = 0.1 ± 0.1
GVH + B6 Kidney 2X	ND[c]	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.3 $\bar{X}$ = 0 ± 0

[a] all animals were grafted with B6 skin on day 20 post GVH induction.
[b] B6 kidney cells were administered I.P on days 13 and 30 post GVH.
[c] Not done.

pletely suppressed. This lack of responsiveness to SRBC cannot result from antigenic competition between B6 and SRBC antigens since normal mice which had been challenged 3 times with B6 antigens showed the same PFC response to SRBC as normal mice which had received only 1 exposure to B6 antigen 23 days earlier.

PFC Response and Hemagglutinating Antibody Titre of GVH Mice Following Multiple Injections with SRBC.

The previous experiments show that GVH mice which had rejected B6 skin grafts and were then injected with SRBC failed to produce any detectable antibodies to either B6 antigens or to SRBC. It seems logical, however, that skin grafting and two injections with B6 kidney cells would present a stronger immunogenic stimulus than a single injection with SRBC. In view of the large number of antigens believed to be present on SRBC, one might question whether several injections with SRBC might be more likely to stimulate a humoral immune response in GVH mice. An attempt was therefore made to detect the presence of circulating antibodies in GVH mice following repeated injections with SRBC (13). GVH mice and a group of normal controls were given intraperitoneal injections of 7×10^8 SRBC on days 13, 20 and 24 after the induction of the GVH reaction. Four days after each injection with SRBC several mice from each group were bled and sacrificed. The number of both direct and indirect PFC in their spleens was determined. Sera were stored at -30°C and within a week the levels of hemagglutinating antibody to SRBC were determined

using the sensitive PVP method.

Four days after the first injection with SRBC normal mice had a mean value of 196.9 x 10^3 direct PFC and 201.5 x 10^3 indirect PFC per spleen (Table 11). In these animals the mean $\log_2$ hemagglutinating end point to SRBC was 8.56. GVH mice receiving one injection of SRBC produced no PFC of either antibody type and had no detectable hemagglutinating antibody to SRBC. After a second injection with SRBC the mean $\log_2$ hemagglutinating end point in normal mice increased to 9.78, while GVH mice showed an insignificant response of 0.44. PFC responses were not determined in these animals. Following the third injection with SRBC normal mice had a mean direct PFC response of 15.8 x 10^3 and an indirect response of 157.4 x 10^3 PFC per spleen. The response in GVH mice was negligible: 1.7 x 10^3 direct and 1.5 x 10^3 indirect PFC. Similarly, normal mice had a mean hemagglutinating end point of greater than 10 while GVH mice had no detectable hemagglutinating antibodies to SRBC.

The use of multiple injections with SRBC provides a strong, immunogenic stimulus which was shown by both PFC and hemagglutination assays to elicit a strong humoral immune response in normal mice (Table 11). It was impossible, however, to detect any significant direct or indirect plaque forming cells in GVH mice after one injection or 3 injections with SRBC. In the present experiment, the use of PVP to augment hemagglutination provides a much more sensitive means of detecting very low levels of circulating antibody which may not be

TABLE 11

Hemagglutinating Antibody Titre (log_2) and PFC Response to SRBC

of Normal and GVH Mice Treated with Multiple Injections of SRBC[a]

Treatment	NORMAL			GVH		
	PFC/spleen X 10^3		Hemagglutinating Titre[b]	PFC/spleen X 10^3		Hemagglutinating Titre[b]
	Direct	Indirect		Direct	Indirect	
+ SRBC 1X	164.4 166.8 167.4 178.8 184.2 204.6 220.2 236.1 249.6 $\bar{X}$ = 196.9 ± 10.7	166.5 168.9 172.8 183.3 190.8 209.4 222.3 240.3 259.2 $\bar{X}$ = 201.5 ± 11.1	6 6 7 9 9 10 10 10 10 $\bar{X}$ = 8.56 ± 0.58	0 0 0 0 0 0 0 0 0 $\bar{X}$ = 0 ± 0	0 0 0 0 0 0 0 0 0 $\bar{X}$ = 0 ± 0	0 0 0 0 0 0 0 0 0 $\bar{X}$ = 0 ± 0
+ SRBC 2X	ND[c]	ND	8 10 10 10 10 10 10 10 10 $\bar{X}$ = 9.78 ± 0.22	ND	ND	0 0 0 0 0 0 0 0 4.0 $\bar{X}$ = 0.44 ± 0.44
+ SRBC 3X	3.6 6.6 6.6 15.0 15.0 15.3 15.6 26.1 38.7 $\bar{X}$ = 15.8 ± 3.6	61.7 69.5 71.6 124.1 132 8 169 7 170.3 262.4 354.2 $\bar{X}$ = 157.4 ± 32.5	>10 >10 >10 >10 >10 >10 >10 >10 >10 $\bar{X}$ = >10	0 0 0 0 0.3 1.2 1.8 2.4 4.8 6.3 $\bar{X}$ = 1.68 ± 0.71	0 0 0 0 0.6 0.6 1.2 1.5 4.5 6.6 $\bar{X}$ = 1 50 ± 0.71	0 0 0 0 0 0 0 0 0 0 $\bar{X}$ = 0 ± 0

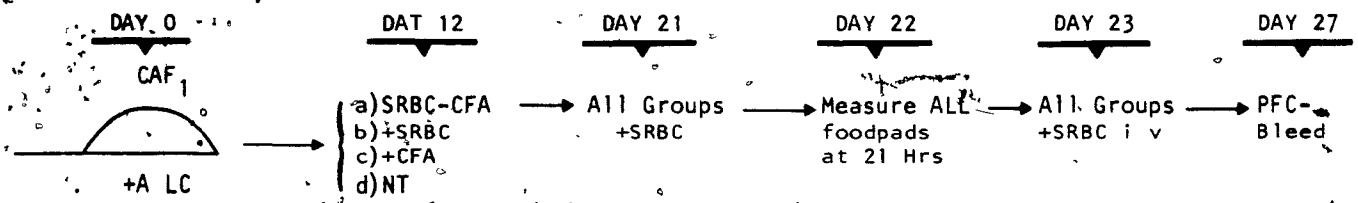
[a]mean values ± SE are given following individual responses for each group.
[b]values represent reciprocal log_2 hemagglutinating titres.
[c]ND - not done

detected with a PFC assay. Using this technique it was not possible to detect any such hemagglutinating antibodies in GVH mice even after 3 injections with SRBC.

Attempts to Stimulate Delayed Hypersensitivity to SRBC in GVH Mice.

Multiple challenges with B6 antigens were effective in eliciting a cell-mediated rejection of B6 skin grafts in GVH mice but failed to stimulate the production of any significant antibody to the same transplantation antigens. Multiple challenges with SRBC also failed to stimulate any detectable humoral immune response in GVH mice. Studies were then carried out to determine if a cell-mediated immune response to SRBC could be stimulated in GVH mice which had been unable to mount a humoral response to SRBC. Following the basic protocol described by Axelrad (1), attempts were made to induce delayed-type hypersensitivity to SRBC in normal and GVH mice.

As shown in Figure 6, GVH reactions were initiated in 4 groups of adult CAF_1 mice by the intravenous injection of 75×10^6 A strain lymphoid cells. On day 12 post GVH induction experimental groups of normal and GVH mice were given an intradermal injection of a SRBC emulsion in Freund's complete adjuvant (SRBC-CFA) in the left footpad. One group of GVH and normal control animals received intradermal injections of 50% SRBC in saline and a second group of GVH and normal animals received an injection of Freund's adjuvant (CFA) in the left footpad. The third control group received no further treatment. Nine



A LC - A Strain Lymphoid Cells; CFA - Complete Freund's Adjuvant; NT - No Treatment;
SRBC-CFA - SRBC in Complete Freund's Adjuvant;

FIGURE 6: Design of Experiment to Stimulate Delayed Hypersensitivity in GVH Mice.

days after sensitization all groups of experimental and control animals were challenged with intradermal injections of 0.1 ml of a 10% solution of SRBC in saline in the right footpad. Using the technique described by Axelbrad (1) and outlined in Chapter 2, measurements of the footpad volume were made immediately before and again 21 hours after challenge with SRBC. Following the final footpad measurement all mice were given an intravenous injection of 5×10^8 SRBC, and 4 days later they were bled and PFC assays performed on their spleens.

Table 12 shows the extent of delayed hypersensitivity in normal and GVH mice 21 hours after challenging with SRBC. The index of delayed hypersensitivity is given as the percentage increase in paw volume of treated animals minus the percentage increase for unsensitized controls. In the group of mice sensitized to SRBC in Freund's adjuvant, normal mice showed a mean increase of 22.9% while GVH mice had a mean increase of 18.7%. Significant but considerably smaller amounts of swelling were observed in normal and GVH mice which had been sensitized to SRBC in saline. For some reason, a significant footpad swelling of 9.3% was observed in normal mice sensitized to Freund's adjuvant and challenged with SRBC: the swelling for GVH animals in this group was somewhat lower but still above the control value. In control mice receiving no sensitizing treatment before challenge with SRBC, a swelling of 2% was observed. These values were subtracted from those observed in the experimental groups to give the index of delayed hypersensitivity for each group.

Although GVH mice showed a delayed hypersensitivity to SRBC which was comparable to that observed in normal mice, no significant

TABLE 12

Delayed Hypersensitivity to SRBC in GVH Mice 9 Days After Sensitization with SRBC in Complete Freund's Adjuvant (CFA)[a,b]

Experimental Group	Sensitizing Antigen	No. of Mice	Mean D.H. (± SE)
Normal	SRBC-CFA	12	22.9 ± 3.2
GVH	"	12	18.7 ± 3.1
Normal	SRBC Alone	8	12.4 ± 1.8
GVH	"	8	3.9 ± 2.6
Normal	CFA Alone	8	9.3 ± 2.5
GVH	"	6	4.1 ± 2.3
Normal	Not Sensitized	8	control[c]
GVH	Not Sensitized	8	control[c]

[a] mice were sensitized with SRBC-CFA 12 days post GVH induction, and were challenged with SRBC alone 9 days later and footpad swelling measured at 21 hours.

[b] delayed hypersensitivity (DH) is indicated as per cent increase in footpad volume of treated animals minus per cent increase for unsensitized controls.

[c] both normal and GVH mice which were not previously sensitized to SRBC exhibited footpad swelling of between 1 and 2%.

humoral response to SRBC could be detected in GVH mice. Table 13 shows that normal mice sensitized to SRBC in Freund's adjuvant followed by intradermal and intravenous challenges with SRBC produced a mean PFC response of 42.6 x 10^3 direct and 136.3 x 10^3 indirect PFC per spleen. The corresponding level ($\log_2$) of circulating antibody to SRBC in these mice was greater than 10. GVH mice receiving the same treatment produced a negligible number of direct and indirect PFC and had no detectable amount of circulating antibody to SRBC. Normal mice which received only one intradermal and an intravenous challenge with SRBC produced a large number of both direct (206.4 x 10^3) and indirect (298.3 x 10^3) PFC, and had a $\log_2$ hemagglutinin titre of greater than 10. GVH mice receiving the same 2 challenges with SRBC showed no significant humoral response to SRBC. The PFC responses and antibody titre were all less than 1% of the values obtained with normal mice.

Hypersensitivity reactions of the delayed type have been shown to be cell-mediated in nature and capable of being transferred with cells from a sensitized animal (2,5). The ability to elicit delayed hypersensitivity in GVH aniamls sensitized with SRBC in Freund's adjuvant suggests that GVH-immunosuppressed animals may be capable of mounting cell-mediated responses to a wide range of xenogenic antigens as well as to allogenic transplantation antigens. This provides further support for our earlier findings suggesting that the GVH reaction exerts a stronger immunosuppressive effect on humoral

TABLE 13

PFC Response and Hemagglutinin Titre to SRBC in GVH Mice Showing Delayed Hypersensitivity to SRBC[a].

Experimental Group	Sensitizing Antigen	Direct PFC ($X 10^3 \pm SE$)	Indirect PFC ($X 10^3 \pm SE$)	Antibody Titre (log_2)
Normal	SRBC-CFA	4.2 11.1 11.7 14.4 33.3 35.4 44.1 46.2 50.4 52.2 66.9 141.3 $\bar{X} = 42.6 \pm 10.6$	15.3 44.1 83.1 85.8 105.6 109.2 112.5 114.0 208.5 219.3 235.5 302.1 $\bar{X} = 136.3 \pm 24.7$	>10 >10 >10 >10 >10 >10 $\bar{X} =$ >10
GVH	"	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.6 2.1 $\bar{X} = 0.2 \pm 0.2$	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.3 $\bar{X} = 0.0 \pm 0.0$	0 0 0 0 $\bar{X} = 0 \pm 0$
Normal	SRBC Alone	22.5 24.9 27.0 35.4 38.4 41.1 53.1 74.7 $\bar{X} = 39.6 \pm 6.1$	102.0 106.2 111.3 137.7 147.0 149.1 230.7 258.9 $\bar{X} = 155.4 \pm 20.7$	>10 >10 >10 >10 $\bar{X} =$ >10
GVH	"	0.0 0.0 0.0 0.0 0.0 0.0 0.6 0.6 $\bar{X} = 0.2 \pm 0.1$	0.0 0.0 0.0 0.3 0.3 0.6 0.9 0.9 $\bar{X} = 0.4 \pm 0.1$	0 0 1 3 $\bar{X} = 1.0 \pm 0.7$

(Continued).....

TABLE 13 Continued.

Experimental Group	Sensitizing Antigen	Direct PFC ($X\ 10^3 \pm SE$)	Indirect PFC ($X\ 10^3 \pm SE$)	Antibody Titre ($\log_2$)
Normal	CFA Alone	161.4 168.9 188.7 196.5 216.9 265.5 267.0 278.4 $\bar{X} = 217.9 \pm 16.5$	186.6 202.5 216.9 229.2 234.0 239.4 257.4 336.0 $\bar{X} = 237.8 \pm 16.0$	>10 >10 >10 >10 $\bar{X} =$ >10
GVH	"	0.0 0.0 0.0 0.0 0.0 0.0 $\bar{X} = 0.0 \pm 0.0$	0.0 0.0 0.3 0.3 0.6 0.6 $\bar{X} = 0.3 \pm 0.1$	0 0 0 $\bar{X} = 0 \pm 0$
Normal	Not previously sensitized	129.3 131.7 145.8 168.9 184.8 201.6 216.3 239.7 241.8 264.9 269.4 282.0 $\bar{X} = 206.4 \pm 15.7$	144.9 154.2 182.4 213.3 252.0 274.5 293.4 327.6 366.0 386.4 468.0 516.9 $\bar{X} = 298.3 \pm 34.5$	>10 >10 >10 >10 >10 >10 $\bar{X} =$ >10
GVH	"	0.0 0.0 0.0 0.0 0.0 0.0 0.3 0.6 0.9 1.2 4.2 $\bar{X} = 0.7 \pm 0.4$	0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.3 0.9 0.9 $\bar{X} = 0.2 \pm 0.1$	0 0 0 2 3 $\bar{X} = 1.0 \pm 0.6$

[a] mice were sensitized with SRBC-CFA 12 days post GVH induction and were challenged with SRBC alone 9 days later. Two days after challenge they received 5 X 10^8 SRBC IV, and hemagglutination and PFC assays were performed 4 days later.

immune responses than on cell-mediated responses to the same antigens.

An interesting trend can be observed from the data presented for normal mice in Tables 12 and 13. Normal animals sensitized with SRBC in complete Freund's adjuvant produced a delayed hypersensitivity response of 22.9%, a value comparable to that reported by Axelrad (1) for mice receiving the same treatment. When these mice were subsequently challenged with an intravenous injection of SRBC they exhibited only a moderate PFC response to SRBC. The direct PFC response in animals experiencing delayed hypersensitivity was only 21% of the direct response observed in normal controls receiving only intravenous SRBC. The corresponding indirect PFC response in hypersensitive mice was approximately 45% that in the controls.

These observations would suggest that an inverse relationship exists between delayed hypersensitivity to SRBC and the PFC response to SRBC. The stimulation of a delayed hypersensitivity response to SRBC appears to reduce the extent of a subsequent humoral response to SRBC. Although this particular effect has not been reported, several investigators (1,4,8) have described the converse situation. The intravenous injection of SRBC prior to primary sensitization with SRBC in adjuvant produced a significant PFC response but caused a marked suppression of delayed hypersensitivity. Furthermore, suppression of delayed hypersensitivity was also brought about by the injection of "7S" anti-SRBC antibody prior to primary sensitization with SRBC in adjuvant. Axelrad (1) initially interpreted this phenomenon as suggesting that the

development of delayed hypersensitivity and direct hemolytic PFC to SRBC may be dependent upon a common immunocompetent precursor cell, and that immunization with SRBC in the absence of adjuvant results in the commitment of such a precursor cell to a pathway leading to humoral immune responses. More recently (4,8) the suppression of delayed hypersensitivity by humoral activity to the same antigen has been explained in terms of an antigen-antibody interaction which blocks the activated T cells which mediate delayed hypersensitivity without blocking the helper cells.

Parish (9) has described a similar inverse relationship between cell-mediated and humoral reactions to a number of antigens. His work suggests that the type of reaction which develops is determined by the form in which the antigen is presented to the antigen reactive cells. The injection of SRBC normally stimulates a strong humoral response. Chemical modification of SRBC by either periodate oxidation or acetoacetylation resulted in preparations which stimulated lower antibody responses than did normal SRBC, but induced much higher levels of delayed hypersensitivity in the absence of Freund's adjuvant.

The present results suggest that in normal animals a delayed hypersensitivity reaction to SRBC reduces the subsequent humoral response to SRBC. In addition, GVH mice developed a significant delayed hypersensivity response to SRBC but showed no detectable humoral response to SRBC. These findings could be easily explained if a common SRBC-sensitive precursor cell were being diverted from a humoral type differentiation to

one for delayed hypersensitivity. The Axelrad proposal therefore remains an attractive hypothesis which may have bearing on the differences in the extent of GVH induced immunosuppression in cell-mediated and humoral immune responses.

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CHAPTER 5: RESULTS

Effect of the GVH Reaction on the Humoral Response to a Thymic-Independent Antigen.

Introduction

Thus far, selective stimulation of cell-mediated immune responses in GVH immunosuppressed mice has been demonstrated using 2 different types of cell-mediated responses to 2 different antigens. It has been shown that appropriate stimulation of GVH mice with specific H antigens will cause a rapid rejection of subsequent skin allografts of the same H specificity. Similarly, appropriate stimulation of GVH mice with SRBC in Freund's adjuvant elicited a delayed hypersensitivity response to SRBC. In both cases, however, it was not possible to detect any humoral response to the same antigens which stimulated the cell-mediated reactions in these mice. Both test systems, however, employed thymic-dependent antigens which require T and B cell interaction in order to stimulate a humoral immune response (4). Although GVH mice could not be stimulated to mount a humoral response to these thymic-dependent antigens, it was not known whether similar stimulation with thymic-independent antigens could elicit humoral responses which do not require T cell cooperation.

Several studies have shown that the GVH reaction suppresses the response to thymic-independent as well as thymic-dependent antigens. Möller (7) has shown that mice sensitized to E. coli endotoxin 7 days

after the initiation of a GVH reaction were unable to mount a humoral immune response to the endotoxin lipopolysaccharide (LPS). Following a single injection with LPS, GVH mice exhibited a PFC response to SRBC coated with LPS which was only 1-5% that seen in normal animals injected with LPS. In a similar study using the thymic-independent type 3 pneumococcal polysaccharide (S III), Byfield et al. (3) reported that GVH mice were totally unresponsive to S III when it was administered in a single dose 6 days after the parental cells.

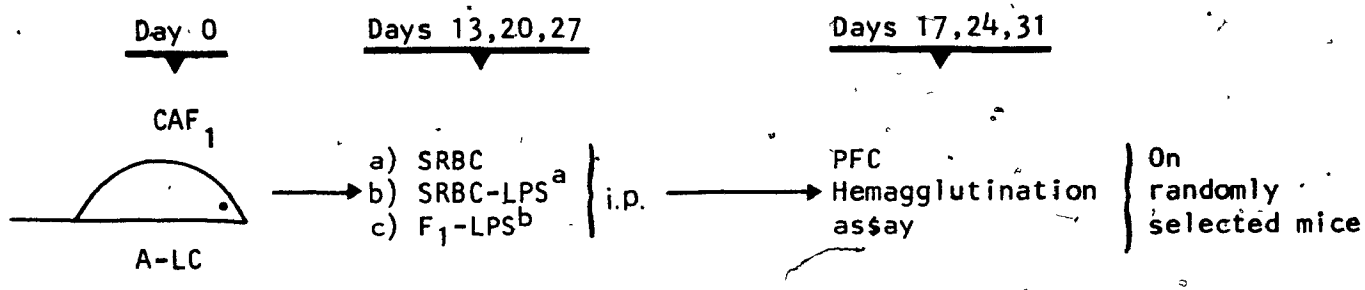
In both of the previous studies antibody production was assayed in GVH mice soon after a single exposure to the thymic-independent antigen. Since multiple exposures to H antigens were effective in stimulating cell-mediated reactions in GVH immunosuppressed mice, it appeared logical to ask whether multiple challenges with a thymic-independent antigen could elicit a humoral response in such mice. Experiments were therefore undertaken to determine if there were any differences in the immunosuppressive effect exerted by the GVH reaction on antibody formation to thymic-dependent versus thymic-independent antigens.

Effect of Multiple Challenges with LPS on Humoral Immune Response.

GVH reactions were induced in 3 groups of adult CAF_1 male mice by the intravenous injection of 75×10^6 A strain lymphoid cells. One group of GVH mice and one group of normal mice received intraperitoneal injections of 7×10^8 SRBC on days 13, 20 and 27 post GVH induction.

The second group of GVH mice and normal controls received similar injections of SRBC coated with LPS (SRBC-LPS) and the third group received syngeneic CAF_1 RBC coated with LPS (F_1-LPS) on days 13, 20 and 27. Four days after each challenge with antigen, mice selected at random from each group were bled by cardiac puncture and their spleens assayed for PFC to both SRBC and SRBC-LPS. Sera were stored at -30° and within one week were assayed for hemagglutinating antibodies to SRBC and SRBC-LPS. The experimental procedure is shown in Figure 7.

Table 14 illustrates the specificity of the anti-LPS response in normal mice following sensitization with endotoxin. Mice sensitized with syngeneic RBC coated with LPS produced a significant number of PFC to LPS when assayed 4 days later. The PFC response to LPS in these animals was virtually the same whether the target cells were F_1-LPS or SRBC-LPS Specificity of this response for LPS is shown by the fact that no significant PFC were observed when the target was SRBC alone. Sensitization with an intraperitoneal injection of 1 mg LPS in 0.5 ml saline produced more than twice as many PFC against SRBC-LPS as did sensitization with F_1-LPS. The PFC response to uncoated SRBC in mice sensitized with LPS alone was only 2% of the PFC response to SRBC-LPS. This slight cross reactivity may result from a non-specific mitogenic effect by LPS at such high immunizing doses. Complete specificity can be assured however, by sensitizing animals with LPS coated to syngeneic cells. No naturally occurring antibodies to LPS could be detected in



[a] SRBC coated with LPS
[b] CAF_1 RBC coated with LPS
A LC - A Strain Lymphoid Cells;

FIGURE 7: Design of Experiment to Determine the Effect on GVH Mice of Multiple Challenges with the Thymic-Independent Antigen LPS.

TABLE 14

Specificity of the PFC Response in Normal Mice Sensitized with LPS[a].

Sensitizing Antigen	Direct PFC per Spleen x 10^3		
	F_1-LPS Target[b]	SRBC-LPS Target[c]	SRBC Target
F_1-LPS[b]	46.8 48.3 58.8 74.4 120.3 186.0 $\bar{X}$ = 89.1 ± 22.3	42.6 50.4 63.6 102.6 104.4 193.8 $\bar{X}$ = 92.9 ± 22.8	0.3 0.3 0.3 0.6 0.6 $\bar{X}$ = 0.4 ± 0.1
LPS	Not Done	173.4 199.2 217.5 245.4 249.6 319.2 $\bar{X}$ = 234.0 ± 20.6	2.7 3.9 4.2 4.5 4.8 5.7 $\bar{X}$ = 4.3 ± 0.4
SRBC	0.0 0.0 0.0 0.0 $\bar{X}$ = 0.0 ± 0.0	243.0 257.7 267.0 269.1 357.3 414.9 $\bar{X}$ = 301.5 ± 28.0	58.5 489.0 619.2 717.0 $\bar{X}$ = 470.9 ± 145
No Treatment	0.0 0.0 0.0 0.0 $\bar{X}$ = 0.0 ± 0.0	0.0 0.0 0.3 0.6 1.2 1.2 1.5 2.1 $\bar{X}$ = 0.9 ± 0.3	0.0 0.0 0.0 0.0 0.0 0.0 0.0 3.0 $\bar{X}$ = 0.4 ± 0.4

[a] mean values ± SE are given following individual values for each group.
[b] CAF_1 RBC coated with LPS.
[c] SRBC coated with LPS.

unsensitized mice either by PFC assay or by hemagglutination.

The data represented in Table 15 and Figure 8 illustrate that the direct PFC responses to both SRBC and to LPS were almost completely suppressed in GVH mice which were sensitized only once with these antigens. While normal mice injected with SRBC produced a very large number of PFC to SRBC, GVH animals receiving SRBC produced less than 1% of the PFC seen in the normal animals. Suppression of the humoral response to LPS was also evident in GVH animals. Normal mice receiving one injection of F_1-LPS produced a significant number of direct PFC to SRBC-LPS while GVH mice treated in the same manner had a direct PFC response which was less than 8% of the value seen in normal animals. The PFC response to F_1-LPS was again confirmed to be specific for LPS, since normal and GVH mice sensitized with F_1-LPS produced no PFC directed against SRBC.

These findings indicate that immunosuppression in GVH animals extends to thymic-independent antigens and that a single challenge with LPS cannot stimulate a humoral immune response in such mice. These results are in agreement with those reported by Möller (7) for GVH mice receiving single injections of LPS. When GVH mice were challenged 3 times with LPS, however, a significant humoral response to LPS could be detected. Table 16 shows that normal mice receiving 3 injections of F_1-LPS produced mean values of 47.7×10^3 direct and 54.7×10^3 indirect PFC to SRBC-LPS. The difference between the direct and indirect responses is not statistically significant. Three treatments of GVH mice with F_1-LPS stimulated a significant direct response to SRBC-LPS of

TABLE 15

Direct PFC Response to SRBC and LPS in Mice

Sensitized Once with the Antigen Indicated[a].

Treatment	Sensitizing Antigen	Direct PFC per Spleen x 10^3	
		SRBC-LPS Target[b]	SRBC Target
Normal	SRBC	354.9 411.9 449.1 462.6 485.4 $\bar{X}$ = 432.8 ± 22.8	252.3 318.6 327.9 332.7 351.6 $\bar{X}$ = 316.6 ± 17.0
GVH	"	0.9 0.9 1.5 1.5 6.9 $\bar{X}$ = 2.3 ± 1.2	0.0 0.0 0.0 0.3 0.3 $\bar{X}$ = 0.1 ± 0.1
Normal	F_1-LPS[c]	36.3 39.0 39.9 57.6 60.9 $\bar{X}$ = 46.7 ± 5.2	0.0 0.0 0.0 0.0 0.0 $\bar{X}$ = 0.0 ± 0.0
GVH	"	0.6 0.9 3.0 9.9 $\bar{X}$ = 3.6 ± 2.2	0.0 0.0 0.0 0.0 $\bar{X}$ = 0.0 ± 0.0

[a] mean values ± SE are given following individual values for each group.
[b] SRBC coated with LPS.
[c] CAF_1 RBC coated with LPS.

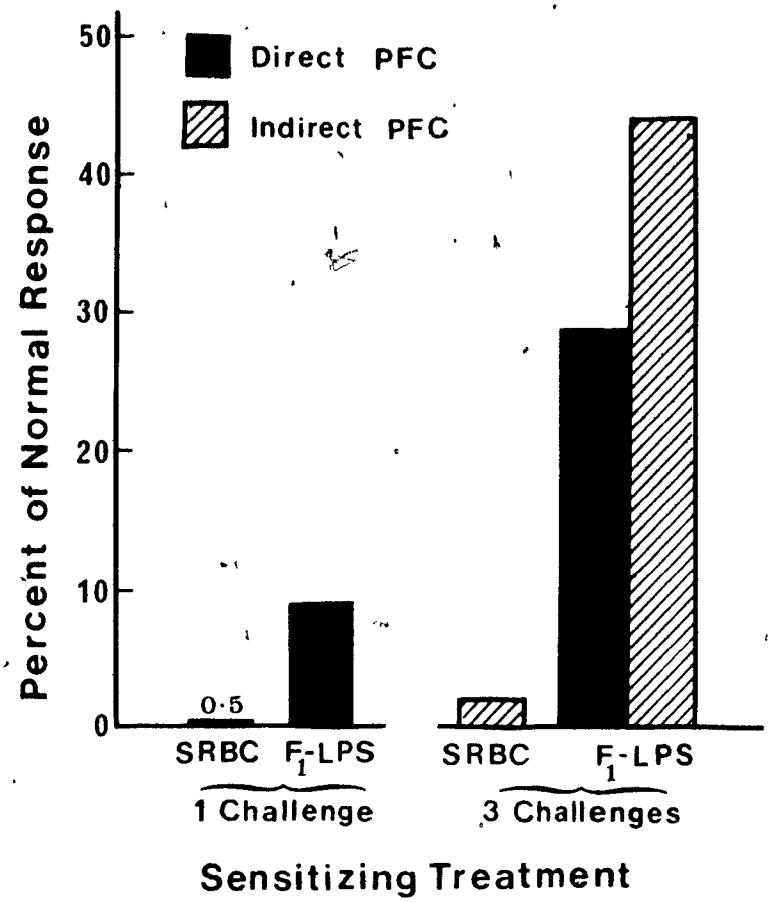


FIGURE 8: PFC Response to SRBC-LPS[a] Target in GVH Mice Sensitized with SRBC or F_1-LPS[b]. (Expressed as Per Cent of Normal Response)[c]

[a]SRBC coated with LPS.
[b]CAF_1 RBC coated with LPS.
[c]% = $\frac{\text{Mean PFC for GVH}}{\text{Mean PFC for Normals}}$ X 100

TABLE 16

PFC Response to SRBC and LPS in Mice Sensitized 3 Times with the Antigens Indicated[a].

Treatment	Sensitizing Antigen	SRBC - LPS[b] Target		SRBC Alone Target	
		Direct PFC X 10^3	Indirect PFC X 10^3	Direct PFC X 10^3	Indirect PFC X 10^3
Normal	SRBC	3.3 10.2 12.3 12.9 21.3 31.5 $\bar{X}$ = 15.3 ± 4.0	87.0 103.1 117.0 166.4 200.1 253.2 $\bar{X}$ = 154.5 ± 26.2	3.6 6.6 6.6 15.0 15.0 15.3 15.6 26.1 38.7 $\bar{X}$ = 15.8 ± 3.6	49.5 62.4 68.7 132.6 147.0 160.5 169.5 261.9 364.5 $\bar{X}$ = 157.4 ± 34.0
GVH	"	0.0 0.0 0.0 0.0 1.5 3.6 8.1 $\bar{X}$ = 1.9 ± 1.2	0.0 0.0 0.6 0.9 3.0 9.3 11.4 $\bar{X}$ = 3.6 ± 1.8	0.0 0.0 0.0 0.0 0.3 1.2 1.8 2 4 4.8 6.3 $\bar{X}$ = 1.7 ± 0.7	0.0 0.0 0.0 0.3 0.6 1.8 5.1 9.0 9.3 10.2 $\bar{X}$ = 3.6 ± 1.4
Normal	F_1-LPS[c]	26.4 40.2 43.5 49.2 79.2 $\bar{X}$ = 47.7 ± 8.7	29.7 45.0 50.7 52.2 95.7 $\bar{X}$ = 54.7 ± 11.0	0.3 0.3 1.2 2.4 4.5 $\bar{X}$ = 1.7 ± 0.8	0.3 0.3 1.2 2.4 5.4 $\bar{X}$ = 1.9 ± 1.0
GVH	"	8.7 10.5 10.8 17.4 21.6 $\bar{X}$ = 13.8 ± 2.5	18.0 19.5 24.3 27.0 32.4 $\bar{X}$ = 24.2 ± 2.6	Not Done	Not Done

(Continued).....

TABLE 16 Continued.

Treatment	Sensitizing Antigen	SRBC - LPS[b] Target		SRBC Alone Target	
		Direct PFC X 10^3	Indirect PFC X 10^3	Direct PFC X 10^3	Indirect PFC X 10^3
Normal	SRBC-LPS[b]	27.6 40.5 50.1 54.9 60.0 60.9 81.3 $\bar{X}$ = 53.6 ± 6.4	111.6 61.0 213.0 225.9 266.1 340.5 455.7 $\bar{X}$ = 253.4 ± 43.6	4.5 6.0 7.2 8.7 9.0 12 6 21.0 $\bar{X}$ = 9.9 ± 2.1	66.6 87.6 122.1 169.5 195.3 230.1 429.6 $\bar{X}$ = 185.8 ± 46.2
GVH	"	0.0 0.6 0.6 2.1 4.8 7.2 9.9 23.1 24.0 $\bar{X}$ = 8.0 ± 3.1	0.6 1.5 3.0 3.0 14.7 22.8 24.6 29.1 39.3 $\bar{X}$ = 15.4 ± 4.7	0.0 0 3 0.3 0.6 4.8 9.3 11 7 12.3 12.6 $\bar{X}$ = 5.8 ± 1.9	0.3 0.3 0.6 1.2 9.3 14.4 16.2 17.4 17.7 $\bar{X}$ = 8.6 ± 2.7
GVH	F_1-LPS + SRBC[d]	5.4 6.0 7.2 9.6 $\bar{X}$ = 7.1 ± 1.0	6.9 13.5 16.2 18.0 $\bar{X}$ = 13.7 ± 2.4	1.2 3.6 4.5 8.1 $\bar{X}$ = 4.4 ± 1.4	4.8 6.3 6.3 14.7 $\bar{X}$ = 8.0 ± 2.3

[a]mean values ± SE are given following individual values for each group.
[b]SRBC coated with LPS.
[c]CAF_1 RBC coated with LPS.
[d]F_1 LPS injected separately from SRBC.

13.8×10^3 and an indirect response of 24.2×10^3 PFC per spleen. The specificity of this response was again shown by the fact that animals sensitized 3 times with F_1-LPS produced no significant PFC to SRBC. Multiple challenges of GVH mice with SRBC-LPS also stimulated a PFC response, although to a lesser degree. From these data it is clear that the humoral response to LPS is not completely suppressed in GVH mice. Three challenges of GVH mice with F_1-LPS stimulated an indirect PFC response which was 44% of that seen in normal mice receiving the same treatment (Figure 8). Multiple stimulation of GVH mice with SRBC, however, produced only negligible indirect responses to SRBC and SRBC-LPS which were less than 2% of the responses in normal animals.

The Cunningham plaque assay permits the identification of specific antibody forming cells which provide an accurate indication of the extent of the humoral response to a given antigen. In cases of low response, however, it is sometimes difficult to distinguish between a high "background" count in unsensitized animals and a low but significant response in experimental animals. In such instances we have found the PVP hemagglutination assay to be antigen specific and to detect very low levels of circulating antibody. When the plaque assay is used in conjunction with the hemagglutination assay, the two provide a sensitive and accurate assessment of the humoral response to a given antigen.

Tables 17, 18 and 19 illustrate the hemagglutinin titres to SRBC and LPS in normal and GVH mice following each challenge with antigen. The data are summarized in Figures 9 and 10. These data reinforce those

TABLE 17

Hemagglutinating Antibody Titre to SRBC and LPS in Mice Sensitized Once with the Antigens Indicated[a].

Treatment	Sensitizing Antigen	Hemagglutinating Antibody Titre (log_2) SRBC-LPS Target	SRBC Target
Normal	SRBC	0 4 6 7 7 8 8 8 8 9 $\bar{X}$ = 6.5 ± 0.8	6 6 7 9 9 10 10 11 11 $\bar{X}$ = 8.78 ± 0.7
GVH	"	0 0 0 0 0 0 0 1 1 $\bar{X}$ = 0.2 ± 0.1	0 0 0 0 0 0 0 0 0 $\bar{X}$ = 0 ± 0
Normal	F_1-LPS[b]	7 8 8 8 9 $\bar{X}$ = 8.0 ± 0.3	0 0 0 0 0 $\bar{X}$ = 0 ± 0
GVH	"	0 0 1 1 2 $\bar{X}$ = 0.8 ± 0.4	0 0 0 0 0 $\bar{X}$ = 0 ± 0
Normal	SRBC-LPS[c]	7 8 8 9 10 10 10 10 11 $\bar{X}$ = 9.2 ± 0.4	6 7 7 8 8 8 9 9 10 $\bar{X}$ = 8.0 ± 0.4
GVH	"	0 0 0 0 0 0 1 2 3 4 $\bar{X}$ = 1.0 ± 0.5	0 0 0 0 0 0 0 0 0 0 $\bar{X}$ = 0 ± 0
GVH	F_1-LPS[d] + SRBC	0 0 2 3 4 $\bar{X}$ = 1.8 ± 0.8	0 0 0 0 0 $\bar{X}$ = 0 ± 0

[a]mean values ± SE are given following individual values for each group.
[b]CAF_1 RBC coated with LPS.
[c]SRBC coated with LPS.
[d]F_1-LPS injected separately from SRBC.

TABLE 18

Hemagglutinating Antibody Titre to SRBC and LPS in Mice Sensitized Twice with the Antigens Indicated[a].

Treatment	Sensitizing Antigen	Hemagglutinating Antibody Titre (log_2) SRBC-LPS Target	SRBC Target
Normal	SRBC	7 9 10 10 10 11 11 12 12 $\bar{X}$ = 10.2 ± 0.5	8 10 10 11 11 11 12 13 13 $\bar{X}$ = 11.0 ± 0.5
GVH	"	0 0 0 0 0 0 0 0 0 $\bar{X}$ = 0 ± 0	0 0 0 0 0 0 0 0 0 $\bar{X}$ = 0 ± 0
Normal	F_1-LPS[b]	10 10 10 10 11 $\bar{X}$ = 10.2 ± 0.2	0 0 0 1 3 $\bar{X}$ = 0.8 ± 0.6
GVH	"	6 7 7 8 9 $\bar{X}$ = 7.4 ± 0.5	0 0 0 0 0 $\bar{X}$ = 0 ± 0
Normal	SRBC-LPS[c]	10 11 11 11 12 12 13 13 $\bar{X}$ = 11.6 ± 0.4	9 10 10 10 11 11 12 $\bar{X}$ = 10.4 ± 0.4
GVH	"	5 6 6 7 7 7 7 8 8 $\bar{X}$ = 6.8 ± 0.3	0 0 0 0 4 4 6 8 $\bar{X}$ = 3.1 ± 1.1
GVH	F_1-LPS[d] + SRBC	6 6 8 8 9 $\bar{X}$ = 7.4 ± 0.6	0 0 0 0 0 $\bar{X}$ = 0 ± 0

[a] mean values ± SE are given following individual values for each group.
[b] CAF_1 RBC coated with LPS.
[c] SRBC coated with LPS.
[d] F_1-LPS injected separately from SRBC.

TABLE 19

Hemagglutinating Antibody Titre to SRBC and LPS in Mice Sensitized 3 Times with the Antigens Indicated[a].

Treatment	Sensitizing Antigen	Hemagglutinating Antibody Titre (log_2) SRBC-LPS Target	SRBC Target
Normal	SRBC	10 10 11 11 11 11 12 12 13 $\bar{X}$ = 11.2 ± 0.3	11 11 11 12 12 12 12 13 13 $\bar{X}$ = 11.9 ± 0.3
GVH	"	0 0 0 0 3 4 5 $\bar{X}$ = 1.7 ± 0.8	0 0 0 0 0 0 0 $\bar{X}$ = 0.0 ± 0.0
Normal	F_1-LPS[b]	10 11 11 11 12 12 $\bar{X}$ = 11.2 ± 0.3	0 0 0 0 0 $\bar{X}$ = 0 ± 0
GVH	"	7 8 10 10 11 $\bar{X}$ = 9.2 ± 0.7	0 0 0 0 0 $\bar{X}$ = 0 ± 0
Normal	SRBC-LPS[c]	10 10 11 12 12 13 13 $\bar{X}$ = 11.6 ± 0.5	9 9 10 10 11 12 12 $\bar{X}$ = 10.4 ± 0.5
GVH	"	8 8 8 9 9 9 10 11 11 $\bar{X}$ = 9.2 ± 0.4	0 0 0 0 1 1 3 4 6 $\bar{X}$ = 1.67 ± 0.7
GVH	F_1-LPS[d] + SRBC	9 10 10 11 $\bar{X}$ = 10.0 ± 0.4	0 0 0 0 $\bar{X}$ = 0 ± 0

[a] mean values ± SE are given following individual values for each group.
[b] CAF_1 RBC coated with LPS.
[c] SRBC coated with LPS.
[d] F_1-LPS injected separately from SRBC.

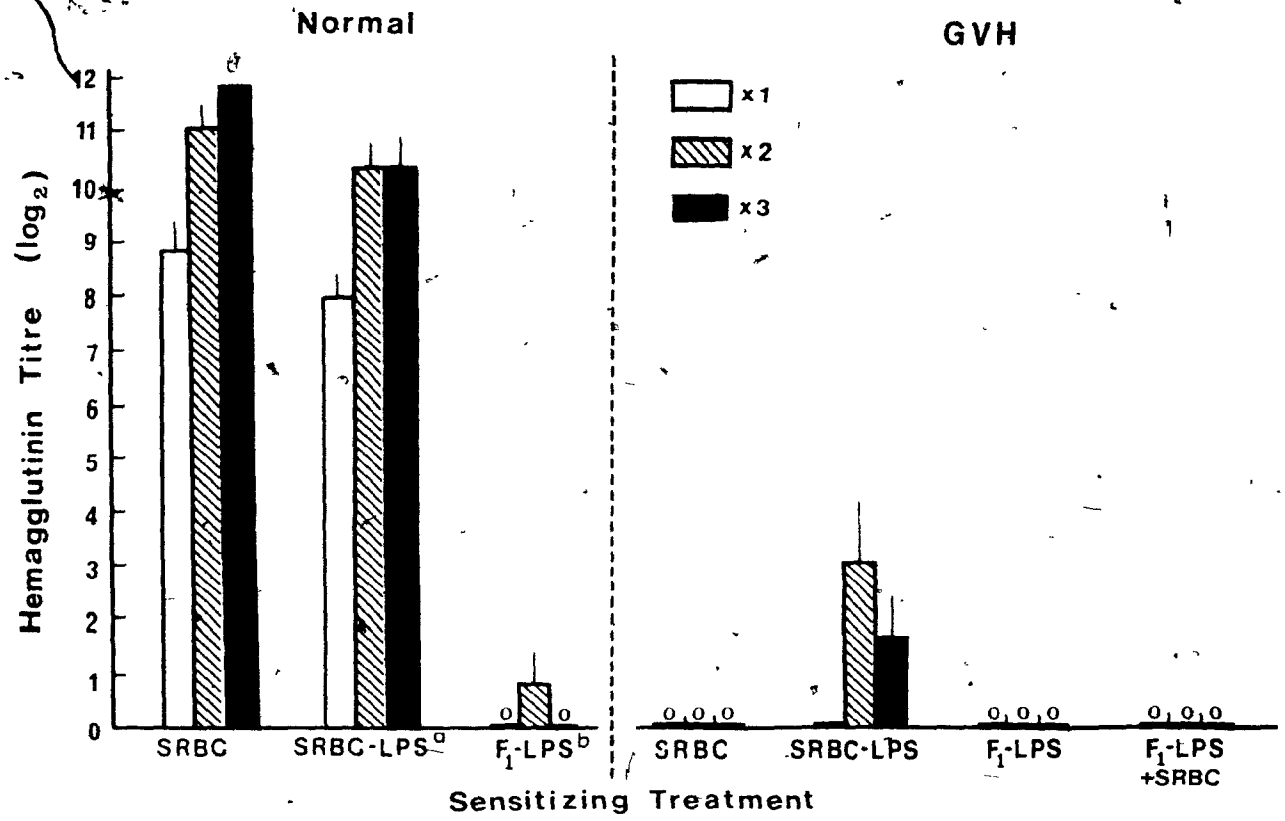


FIGURE 9: Effect of Multiple Stimulation of GVH Mice with LPS. Hemagglutinating Antibody Titre to SRBC.

[a]SRBC coated with LPS.
[b]CAF_1 RBC coated with LPS.

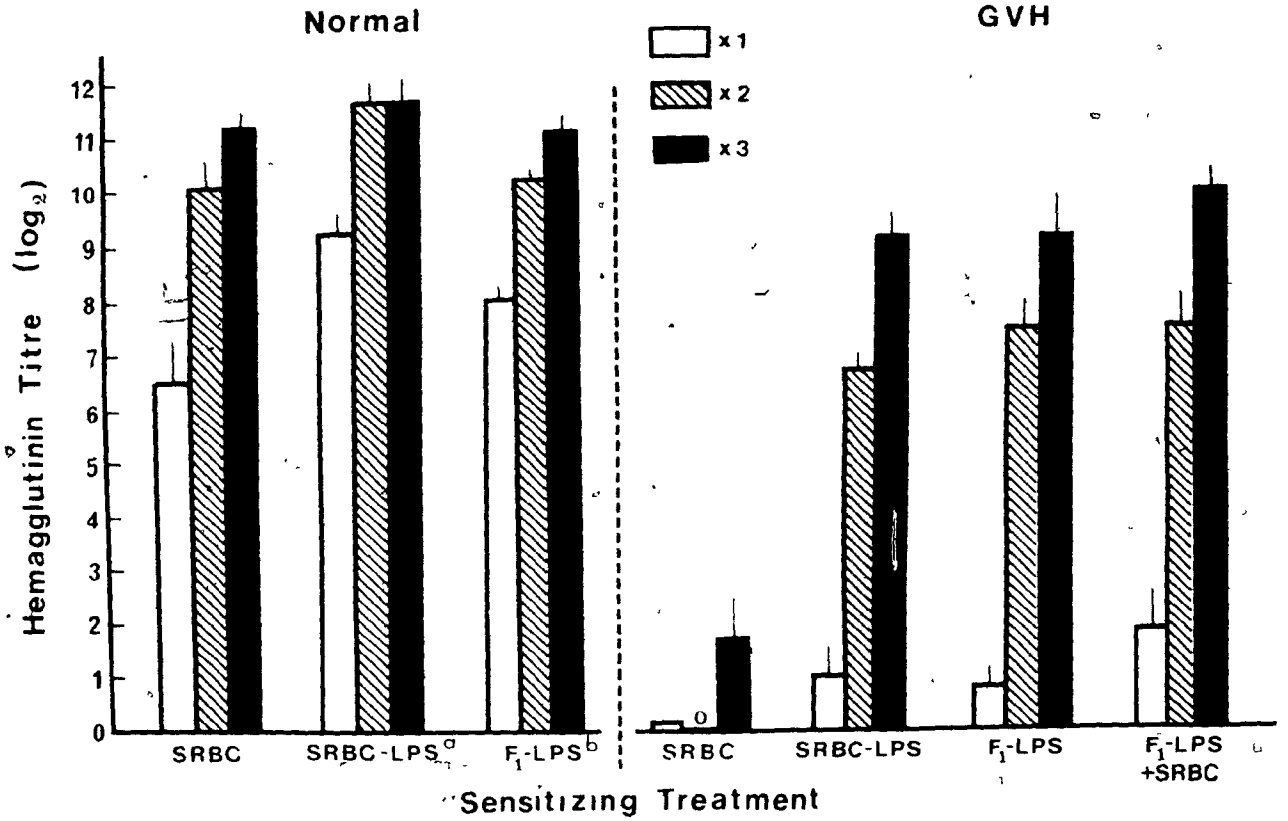


FIGURE 10: Effect of Multiple Stimulation of GVH Mice with LPS. Hemagglutinating Antibody Titre to SRBC-LPS[a].

[a]SRBC coated with LPS.
[b]CAF_1 RBC coated with LPS.

obtained using the PFC assay and provide a more sensitive assessment of the humoral response to both SRBC and LPS. Normal mice had high titres of hemagglutinins to SRBC and SRBC-LPS after a single injection with SRBC. These titres increased following the second injection with SRBC, and after the third injection the hemagglutinin titre to SRBC was 11.9 while the titre to SRBC-LPS was 11.2. A single injection with SRBC-LPS produced a hemagglutinin titre to SRBC-LPS which was larger than the titre produced by a single injection with SRBC. Following the second and third injections with SRBC-LPS the hemagglutinin titres to SRBC-LPS were virtually the same as in normal animals sensitized to SRBC. Sensitization of normal mice with F_1-LPS produced an antibody titre against LPS of 8.0 following the first injection. Following the second and third injections with F_1-LPS the anti-LPS titres rose to 10.2 and 11.2 respectively. The specificity of this antibody was shown by the fact that serum from mice sensitized with F_1-LPS did not agglutinate SRBC.

GVH mice sensitized with SRBC produced no significant amount of antibody capable of agglutinating either SRBC or SRBC-LPS. After 3 challenges with SRBC the levels of hemagglutinating antibodies were less than 0.5% of the levels produced in normal animals receiving the same treatment. GVH mice receiving a single injection of F_1-LPS produced no significant amount of antibody to LPS. Two injections with F_1-LPS, however, stimulated the production of an anti-LPS titre of 9.2. Although GVH mice sensitized with SRBC produced no significant amount

of antibody capable of agglutinating SRBC-LPS, those mice challenged with SRBC-LPS had agglutination titres to SRBC-LPS comparable to those for GVH animals receiving F_1-LPS.

The data obtained from hemagglutination and plaque assays complement each other and point to several conclusions which may be drawn regarding the reaction of GVH mice to multiple stimulation with the thymic-independent antigen, LPS. The present findings support those of Möller (7) indicating that CAF_1 GVH mice are unable to mount a humoral immune response to LPS after a single injection with the endotoxin. Multiple challenges of GVH mice with LPS, however, stimulated a significant PFC response and the production of a large amount of antibody directed against LPS.

The fact that no significant humoral response to LPS could be detected in GVH mice following a single injection with endotoxin suggests that some general impairment of B cell function exists in animals experiencing GVH reactions. Unlike the humoral response to SRBC, this impairment could be overcome by a second injection of endotoxin, resulting in the production of specific antibody against LPS. Although the present study does not show clearly how the humoral response to LPS is stimulated, several suggestions may be made.

In addition to acting as a thymus-independent antigen, LPS has been shown to have mitogenic properties capable of activating B cells directly into increased DNA synthesis (1). Some authors have suggested that the thymus-independent character of LPS derives from its ability to act as a B cell mitogen, but there is a fundamental difference between these 2 pro-

perties exhibited by LPS The ability to stimulate antibody production without cooperation from T cells is seen both in vivo and in vitro when low concentrations of LPS are used (2,5,6). The action is highly specific producing antibody only to LPS. On the other hand, the mitogeneic property has been observed in vitro when large amounts of LPS are added to the culture. This action is non-specific and stimulates clones of cells producing antibodies to a wide range of antigens.

In the present experiments, multiple challenges of GVH mice with LPS resulted in the production of antibody specific for LPS. Sera from stimulated animals had high agglutinin titres to SRBC-LPS but failed to agglutinate SRBC. In view of the specificity of antibody production, it would appear that stimulation of a humoral response to LPS in GVH mice was achieved because of its thymic-independent nature rather than its mitogenic potential. On the other hand, multiple stimulation with thymus-dependent antigens failed to elicit humoral immune responses in GVH mice. These facts would imply that suppression of the humoral response to thymus-dependent antigens in GVH animals is at least in part caused by an impairment of the cooperation necessary between T and B cells for antibody production. Further speculation as to the cause of this suppression will be reserved for a general discussion later.

In one experiment (Table 20), an interesting effect was observed in GVH animals receiving two injections of SRBC-LPS. The four animals in this group produced an antibody titre of $6.3 \pm 0\ 6$ against SRBC-LPS. In addition, these animals also produced a titre of 5.5 ± 1.0 against

TABLE 20

Hemagglutinating Antibody Titre to SRBC in One Experiment in which GVH Mice Were Sensitized Twice with SRBC-LPS[ab].

Treatment	Sensitizing Antigen	Hemagglutinating Antibody Titre (log_2) SRBC-LPS Target	SRBC Target
Normal	SRBC	7 9 10 10 $\bar{X} = 9.0 \pm 0.7$	8 10 10 11 $\bar{X} = 9.8 \pm 0.6$
GVH	"	0 0 0 0 $\bar{X} = 0 \pm 0$	0 0 0 0 $\bar{X} = 0 \pm 0$
Normal	SRBC-LPS[c]	11 12 12 13 $\bar{X} = 12.0 \pm 0.4$	10 10 11 12 $\bar{X} = 10.8 \pm 0.5$
GVH	"	5 6 6 8 $\bar{X} = 6.3 \pm 0.6$	4 4 6 8 $\bar{X} = 5.5 \pm 1.0$
GVH	F_1-LPS[d] + SRBC	6 6 8 8 9 $\bar{X} = 7.4 \pm 0.6$	0 0 0 0 0 $\bar{X} = 0 \pm 0$

[a]mean values ± SE are given following individual values for each group.
[b]data is from one of two experiments represented in Table 18.
[c]SRBC coated with LPS.
[d]CAF_1 RBC coated with LPS and injected separately from SRBC.

SRBC, although no such antibody could be detected in GVH mice receiving SRBC alone. If valid, these results would suggest that LPS coated to SRBC has the ability in GVH mice to stimulate a humoral immune response to the thymic-dependent antigens on SRBC. A somewhat related effect of LPS was reported by Möller et al. (8) in mice which had been thymectomized, irradiated and repopulated with syngeneic bone marrow. Such mice could not mount a PFC response to SRBC when injected with SRBC, but produced an anti-SRBC response that was almost normal when they were injected with SRBC-LPS. It was postulated that this effect was caused by LPS substituting for T cells in the antibody response to SRBC by virtue of its mitogenic effect on B cells. Möller et al found that LPS must be bound to the SRBC to cause this effect, since injecting LPS and SRBC separately failed to restore the response to SRBC. Likewise, in the present experiment, no anti-SRBC antibody could be detected in GVH mice given separate injections of SRBC and LPS.

Unfortunately, LPS stimulated a humoral response by GVH mice to SRBC in only one experiment. Several attempts to reproduce this effect were unsuccessful. It is possible, however, that the interval at which the SRBC-LPS is injected into GVH mice may be critical for restoration of the humoral response to SRBC. Britton and Möller (2) have observed a cyclical pattern in both the PFC response and antibody titre to LPS in normal mice sensitized once to the endotoxin. Their findings suggested that this pattern resulted from a neutralization of LPS by newly formed antibody. As antibody was formed against LPS, it was believed to bind to

antigenic sites on the LPS. As more and more of the circulating LPS became bound, its immunogenicity was lost and the humoral response to it declined. Since it was shown that LPS was not readily metabolized while the antibody had a limited half life, Britton and Möller proposed that the coating antibody was eventually lost and the antigenic determinants on LPS were once again exposed. This result in a reappearance of the humoral response to LPS and the cyclical pattern of antibody formation.

If antibody formation against LPS occurs by a mechanism such as that proposed by Britton and Möller, the timing of a second injection with LPS would be a critical factor in the subsequent response to LPS. If the second injection were given at a time when the anti-LPS antibody were high, the newly injected LPS would be rapidly neutralized and no subsequent humoral response would be detected. A similar mechanism might explain why it is sometimes possible to stimulate antibody formation to SRBC in GVH mice given multiple injections with SRBC-LPS. If SRBC-LPS are injected when the anti-LPS titre is low, enough mitogenic sites on the LPS may remain exposed to assist in a B cell response to SRBC. On the other hand, if SRBC-LPS are injected when there is a high titre of antibody against LPS, these mitogenic sites may become covered and unable to assist in the production of antibody against SRBC.

CHAPTER 5: REFERENCES

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CHAPTER 6: DISCUSSION

Introduction

The results reported here demonstrate that the GVH reaction suppresses both cell-mediated and humoral immune responses. The extent of immunosuppression was shown to depend upon the strength of the ongoing GVH reaction and its duration at the time of sensitization. Adrenalectomy and castration prior to induction of the GVH reaction did not alter the immunological unresponsiveness of the F_1 host, suggesting that immunosuppression results from immunological factors rather than from a general stress response.

Appropriate stimulation of GVH mice was capable of eliciting two different types of cell-mediated immune reactions to two different types of antigen. The humoral immune response to the same antigens remained suppressed however, suggesting that GVH induced suppression of the humoral immune response is more intense than the suppression of the cell-mediated response. Multiple challenges of CAF_1 GVH mice with B6 allotransplantation antigens caused a rapid rejection of subsequent B6 skin grafts but failed to stimulate the production of any detectable antibodies to B6 tissue. Sensitization of GVH mice with SRBC in Freund's adjuvant stimulated a delayed hypersensitivity response to SRBC although no humoral response to SRBC could be detected, even after 3 challenges with SRBC.

It was demonstrated that the humoral response to the thymic-independent antigen, LPS, was also suppressed. It was possible, however,

to activate a humoral response to LPS using multiple challenges with the antigen, a finding similar to that observed for cell-mediated reactions to thymic-dependent antigens.

Interpretation of individual observations has already been made following each section of observations. Collectively, these results can be used to further our understanding of the mechanism of GVH induced immunosuppression as well as the normal regulation of cell-mediated and humoral immunity. Based on other experiments performed in this laboratory, a model will first be presented to explain the immunosuppressive effect of the GVH reaction on humoral immune responses. An analysis of the present findings will be made in terms of the proposed model for humoral immunosuppression. A new model will then be proposed as a possible mechanism for the regulation of both humoral and cell-mediated immune responses in normal animals, and an attempt will be made to explain the immunosuppressive effects of the GVH reaction using this model. Finally, a clinical application will be suggested for the present experiments.

Proposed mechanism for GVH induced immunosuppression.

Early investigations (8,9,32,47,80) suggested that the GVH reaction may bring about immunosuppression by an immunological attack of competent donor cells on the lymphoid cells of the recipient. This would cause a destruction of host lymphoid tissue leading to a depletion or inactivation of cells capable of mounting an immune response. Lawrence

and Simonsen (59) then accounted for GVH induced immunosuppression by proposing that immunologically competent cells were pluripotent and that all cells became activated to host antigens during a GVH reaction, leaving no cells to react to other antigens when they were introduced. This view was discredited somewhat later when Lapp and Möller (56) found that adoptive transfer of normal parental or F_1 lymphoid cells into an F_1 GVH animal did not restore immunocompetence. Their results suggested that the environment in the hybrid host is responsible for immunosuppression, but they did not indicate whether suppression resulted from the production of an inhibitory factor or from competition for a limited amount of some factor which is essential for immune responsiveness.

More recently, the in vitro work of Sjöberg (81,82) and the in vivo studies of Möller (63) suggest that an inhibitory factor may be produced by cells undergoing a GVH reaction. When spleen cells from 7 day GVH mice were cultured with SRBC or LPS, the primary antibody responses against both SRBC and LPS were markedly suppressed. Furthermore, when spleen cells from GVH mice were added to cultures of normal spleen cells the PFC response of the normal cells to SRBC was markedly inhibited. The inhibitory effect of the GVH cells on normal cells was not sensitive to treatment with anti-theta serum and complement, but was completely abolished when adherent cells were removed from the GVH spleens with iron powder. This would suggest that macrophages are directly responsible for suppression. Macrophages are known to be activated during

a GVH reaction (9) and T cells have been shown to provide the major stimulation for macrophage activation (30,31,61,92). It is possible, therefore, that T cells stimulated by the foreign H antigens during a GVH reaction activate macrophages which in turn are responsible for the inhibitory effect of the GVH reaction on the immune response.

Evidence obtained in this laboratory suggests that GVH induced immunosuppression may be caused both by the production of substances which may have an inhibitory effect, and by competition for limited amounts of essential factors. This evidence indicates that immunosuppression results, at least in part, from some defect in the activity of the thymus derived cell population (42,55,58). The administration of thymic tissue or cell free extracts to GVH immunosuppressed mice was able to restore immunocompetence to 55% of the control value (55,58). Suppression was not caused by a lack of T cells per se, since treatment of GVH spleen, thymus, and lymph node cells with an anti-theta serum revealed very little change in T cell number. Since immunocompetence could be partially restored using a cell-free thymic extract, it appeared that the GVH reaction functionally depletes both host and donor T cells of a thymic humoral mediator that is essential for immunocompetence.

The hypothesis just described has also been supported by numerous in vitro restoration experiments performed in this laboratory (26,57,73). Spleen cells were cultured in a modified Marbrook culture vessel consisting of two culture chambers, a reacting chamber and a restoring

chamber, separated by a cell-impermeable membrane. When normal spleen cells were cultured with SRBC in the reacting chamber a significant PFC response to SRBC developed within 4 days. When spleen cells from GVH animals were cultured with SRBC in the same manner, there was no PFC response to SRBC. The PFC response of GVH spleen cells could be significantly restored, however, by adding normal thymus, spleen or lymph node cells to the restoring chamber. Restoration was equally as effective when the thymus, spleen and lymph node cells were from F_1 mice or from either parental strain. A comparable restoration was also obtained when supernatants from cultures of F_1 thymus and lymph node cells were added to the inner chamber. Restoration was even obtained when thymus and lymph node cells from GVH animals were added to the inner chamber. In all cases, treatment of the "restoring" cells with anti-theta serum abrogated their ability to restore the PFC response of GVH spleen cells. These results suggest that GVH induced immunosuppression is caused by a deficiency of a non-specific T cell factor which is most likely essential for some critical event in the activation of B cells.

The findings of a T cell deficiency during GVH immunosuppression are not inconsistent with other reports (65,81,82) of inhibitory substances operating during GVH reactions. As mentioned earlier, reports of inhibitory substances center around the ability of macrophages from GVH spleens to inhibit the PFC response of normal spleen cells. It has been demonstrated recently by us that the production of an essential T cell mediator is regulated by macrophages and that suppression of the humoral immune response in GVH animals appears to be caused by the feedback of

a macrophage factor on the T cells (28,57). The in vitro PFC response of GVH spleen cells to SRBC was restored when thymus or lymph node cells from GVH mice were cultured in the restoring chamber of the modified Marbrook vessel and separated from the reacting cells by a cell-impermeable membrane. Restoration was not obtained when spleen cells from GVH mice were added to the restoring chamber. The removal of adherent cells from the GVH spleen cells in the restoring chamber, however, resulted in almost complete restoration of the PFC response of the GVH spleen cells in the reacting chamber. Furthermore, if the nonadherent GVH spleen cells used to restore the PFC response were treated with anti-theta serum before being placed in the inner chamber, the restorative effect was completely lost. It was concluded, therefore, that suppression of the humoral immune response in GVH animals results from the lack of a soluble factor produced by T cells, and that the release of this factor appears to be controlled by macrophages.

Care must be taken to avoid equating adherent cells with macrophages. Identification of adherent cells as macrophages can be confirmed by examination of morphological and phagocytic characteristics. The ability of adherent cells to phagocytose iron particles correlates well with the ability to adhere to glass and plastic (28,57), suggesting that the large majority of adherent cells are macrophages. Nevertheless, recent evidence suggests that B lymphocytes and even some T lymphocytes may also have adherent characteristics (33). Although it is most likely that effects produced by purified adherent cells result from macrophage activity, one cannot rule out the possibility that such effects results

from the presence of small numbers of B or even T cells.

It has been demonstrated that the proportion of macrophages in the spleens of mice experiencing a GVH reaction gradually increases as the GVH reaction progresses (27,28). The proportion of macrophages in the spleens of $B6AF_1$ mice increased within 2 days after receiving A strain lymphoid cells, and by day 10 post GVH induction the number of macrophages was 8 to 10 times greater than normal. In the present experiments the gradual onset of immunosuppression in GVH mice correlates with the gradual increase in the splenic macrophage population and lends support to the belief that GVH induced immunosuppression results in part from an increase in macrophage activity during the GVH reaction.

Recent studies by Elie and Lapp (27,28) have shown that the role of adherent cells in humoral immune responses is not limited to inhibition, but rather is one of general regulation. The removal of adherent cells from normal spleen cells resulted in their inability to develop a primary PFC response to SRBC in vitro. The PFC response could be restored, however, by the addition of an appropriate number of adherent cells from the spleens of GVH mice. If an excessive number of either GVH adherent cells or normal adherent cells were added to the non-adherent cells from normal spleens, the PFC response was suppressed. These results strongly suggest that suppression is caused by either an active suppression of T cells by adherent cells or by an inactivation of a T cell product by large numbers of adherent cells.

The present experiments demonstrate that B cells are also suppressed during a GVH reaction, since GVH mice were unable to mount a humoral re-

sponse to a single injection of LPS. The suppression of B cells is not as intense, however, as the suppression of T cells since multiple challenges of GVH mice with LPS overcame the B cell defect and stimulated a humoral response to LPS while similar multiple challenges with SRBC could not stimulate a humoral response to SRBC.

From the results presented here, a model has been proposed to explain normal control of the humoral immune response as well as GVH-induced immunosuppression (26). It is generally agreed that 3 types of cells (T cells, B cells, and adherent or A cells) are involved in the normal immune response. It is believed that a B cell requires at least two signals for activation into an expanded pool of antibody forming cells. One signal is provided by antigen which triggers antibody production, and the second is a mitogenic signal which triggers B cell proliferation. T cell factors probably function alone or in concert with other non-specific factors to trigger mitogenesis. Recent evidence suggests that an A cell product as well as a T cell factor which has been processed by A cells may be required for activation of B cells (46,75,91). The model for humoral immune responses proposes therefore that T cell factor (TF) interacts with A cell factor (AF) to form a complex (T-A). The T cell factor is then able to bind to B cells via the AF portion of the T-A complex. Binding of the T-A complex to a mitogenic site on the B cell, as well as the binding of the specific antigen, is necessary for B cell activation. If the uncomplexed AF were present in excess it could have a dual suppressive effect by feeding back to turn off T cells as well as by paralyzing B cells.

From the above model it is proposed that the early immunosuppression observed in GVH animals results from the depletion of T cell factor by the intense reaction of cells responding to the GVH reaction. As the GVH reaction procedes and the A cell population increases, an increase in the amount of A cell factor would cause the uncomplexed AF to bind to the B cell and block its mitogenic site. The high concentration of AF would also complex with any TF as soon as it was released, but binding of the T-A complex and B cell activation would be effectively blocked by the high concentration of uncomplexed A factor already bound to the mitogenic site. This would lead to the long-lasting immunosuppression observed during GVH reactions. This model would account for the findings that the addition of T cell factor restores the PFC response of GVH animals and the removal of adherent cells from GVH spleens blocks the immunosuppressive effect of the GVH spleen cells on normal spleen cells.

The response of GVH mice to multiple injections with LPS can be explained in terms of the proposed model for immunosuppression. Whereas thymic-dependent antigens appear to bind to B cells only at specific antigenic sites, LPS is believed to bind both at specific antigenic sites and at non-specific mitogenic sites (22,23). The binding of LPS and stimulation at the mitogenic site is believed to account for its thymic-independent nature as well as its mitogenic effect. A single injection of F_1-LPS would present the GVH host with a small amount of antigen which would be expected to bind to antigenic sites on specific B cells. At these low concentrations, LPS would be unable to bind to mitogenic sites since these sites would be already bound with the ex-

cess adherent cell factor produced during the GVH reaction. As a result of this competitive inhibition, a humoral response would not be mounted against LPS. Since LPS has been shown to maintain its immunogenicity in vivo for unusually long periods of time (12,13), subsequent injections of LPS would result in the accumulation of a high concentration of LPS. At these higher concentrations, LPS would effectively compete with A cell factor and displace it from the mitogenic sites, thus stimulating antibody formation. A specific anti-LPS response would be produced, since the concentration of LPS would still remain considerably below the levels necessary for the non-specific mitogenic effect (23).

Although repeated stimulation with LPS elicited a humoral response to LPS, multiple challenges with SRBC failed to stimulate a humoral response to the thymic-dependent SRBC. This suggests that, in addition to the B cell defect, there is a functional defect in T cell activity which we believe is caused by A cell factor turning off the release of T cell factor. The possibility that the loss of T cell factor results from over utilization by the cells engaged in the GVH reaction would also fit within the proposed model.

The model which has been presented to account for GVH induced immunosuppression is supported by several lines of evidence obtained in other laboratories. Evidence for macrophage-T cell interaction in the regulation of immune responses during a GVH reaction has been obtained by Scott (78). GVH reactions were initiated in CBA x C57 mice by the injection of C57 lymphoid cells. The spleens from these GVH animals were examined 8 days later and found to be completely unresponsive

to phytohemagglutinin (PHA). Removal of glass adherent cells from the GVH spleens resulted in the recovery of significant PHA responsiveness. The addition of 8 day GVH spleen cells to normal F_1 spleen cells caused an 80% inhibition of C^{14}-thymidine incorporation by the normal lymphocytes following stimulation with PHA. These findings would suggest that GVH-activated macrophages may contribute to immunosuppression by a direct inhibition of normal T cell activities.

Studies using the mixed lymphocyte reaction (149) in vitro graft reactions (62), and the in vitro response to SRBC (45,46) have also demonstrated a biphasic effect exerted by macrophages upon the immune response. Although glass adherent cells are necessary for these reactions, they have been shown to be inhibitory when present in excess. For example, restoring macrophages to glass absorbed spleen cells in a ratio of 1 macrophage for every 50 spleen cells produced a maximum PFC response when these cells were cultured with SRBC. Increasing the ratio to 1 macrophage for very 5 spleen cells caused a marked suppression of the subsequent PFC response to SRBC (45). Subsequent studies (46) showed that this effect could be obtained by using high and low concentrations of a soluble factor produced by peritoneal exudate cells which are mostly macrophages. If a factor produced by activated macrophages does have such a biphasic effect on the immune response, it would explain the seemingly contradictory findings of increased immune responsiveness and immunosuppression observed during GVH and MLC reactions.

Interpretation of the present experiments in terms of the proposed model for GVH induced suppression of the humoral immune response.

The model which has been presented to explain GVH induced immunosuppression is based primarily on *in vivo* and *in vitro* experiments restoring the PFC response of GVH spleen cells to SRBC. The PFC response is a humoral immune response requiring a helper function which is thought to be brought about by soluble factors produced by specific T cells. It is not known, however, whether a thymic mediator also plays a helper role in cell-mediated immune responses. Care must therefore be taken when applying this model to the suppression of cell-mediated responses. The fact that cell-mediated reactions can be selectively stimulated in GVH animals illustrates a basic difference between the two types of immune responses. The present experiments, therefore, serve to provide additional information regarding the factors causing GVH induced suppression of cell-mediated immune responses. Homograft rejection and hypersensitivity reactions of the delayed type have been shown to be cell-mediated in nature and closely related to one another (7,11,17,54 90). If one considers the normal immune reflex arc, there are several points at which cell-mediated reactions could be blocked during a GVH reaction. The afferent limb requires a functional antigen reactive cell to specifically recognize antigen. T cells have been shown to be essential in cell-mediated immune responses and most evidence indicates that the antigen reactive cell is a T cell in both allograft rejection (4,51) and delayed hypersensitivity reactions (20,21). The macrophage also appears

to play a key role in these reactions by processing antigen and presenting it in a stimulatory form to T cells (7,77,90). In fact, the induction of delayed hypersensitivity or antibody production to a given antigen appears to be at least partly determined by the size of the antigen molecule (48,74,84). The effector cell has been shown to be thymus dependent (34) although it has not been established whether it is thymus derived. Studies by Lohmann-Matthes and Fischer (61) and by Golstein et.al. (41) suggest that T cells are capable of recognizing foreign tissue antigens and killing the cells bearing these antigens without cooperation from other cell populations. Other studies (30,31,61,67, 90) suggest that, following the contact of specific T cells with antigen, substances are released which confer upon macrophages the ability to destroy specific target cells. Still other studies (34,43) suggest that B cells may serve as effector cells in cell-mediated reactions.

During a GVH reaction, it is highly likely that the attack upon the host's immune system results in some functional damage at each point in the immune reflex. However, the fact that B6 BM and kidney cells stimulated rapid allograft rejection when administered to animals undergoing a GVH reaction indicates that antigen-reactive cells (most likely T cells) persist in the immunosuppressed animal (86,87,88). Macrophages are also known to be present. Similarly, the stimulation of a specific humoral response with multiple challenges of the thymic-independent LPS indicates that significant numbers of B cells survive as well. It thus would appear that GVH animals possess significant numbers of the

basic cell types necessary to mount both cell-mediated and humoral immune responses. Although it cannot be definitely stated whether these cells are of parental or F_1 origin, most evidence would suggest that they are host cells. In the later stages of GVH reactions of moderate strength, such as in the present study, donor cells have been shown to account for only about 1% of the proliferating cells in the host spleen (35,36,93). It would therefore appear that some form of active suppression is operating for both cell-mediated and humoral immunity, and this effect appears to be mediated by a suppression of T cell function.

It is significant that stimulation with the third party B6 antigen after the induction of the GVH reaction was able to activate enough cells to effect a rapid rejection of subsequent B6 skin grafts. In experiments by Lapp and Möller (56) F_1 mice were sensitized to a third-party skin 30 days before inducing a GVH reaction with parental strain lymphoid cells. When these GVH animals were then grafted with skin allografts from the same third-party strain, the grafts survived significantly longer than identical grafts placed on nonsensitized GVH animals. The difference between the results of Lapp and Möller (56) and the present findings might possibly be attributable to the involvement of physiologically different cell types in sensitized and nonsensitized animals that react to an antigenic challenge. Exposure of a normal animal to foreign histocompatibility antigens results in the production of memory cells which differentiate from the cell line which

reacted to the antigen. These memory cells may well be more susceptible to the immunosuppressive effects of the GVH reaction than antigen-reactive cells from a non-sensitized animal. In the present experiments the GVH reaction was initiated without prior sensitization to histocompatibility antigens. If the non-primed antigen reactive cells are in fact less vulnerable to the suppressive effects of the GVH reaction than sensitized memory cells, then these virgin cells would be able to react to the high concentration of histocompatibility antigens when B6 BM or kidney cells were administered 13 days later.

Stimulation of CAF_1 GVH mice with B6 BM resulted in a more rapid rejection of subsequent B6 skin grafts than did stimulation with B6 kidney cells. Part of the explanation may lie in the fact that the sensitizing BM cells were administered intravenously while the kidney cells were injected intraperitoneally. It is also possible that the B6 BM may serve as a source of innocent bystander cells which are activated by T cells to become killer cells. This is unlikely, however, since the injection of F_1 BM did not significantly alter the degree of GVH induced immunosuppression as measured by skin graft survival time. The MST of skin grafts on the group that received F_1 BM was longer than for the group that received a GVH alone, but this difference was not statistically significant (Table 6).

Although the increased MST for B6 skin grafts made on CAF_1 GVH mice receiving F_1 BM is not statistically significant, the work of Streilein (83) would suggest that such a trend is nonetheless, likely.

Streilein noted that the GVH reaction itself subsides following the early period of rapid cell proliferation. He attributed this to a depletion of F_1 lymphoid cells which were acting as target cells for the GVH reaction. He postulated that the injection of F_1 BM into such an animal would replenish the target cells and restore the GVH reaction. If such is the case, one might expect that the restored GVH reaction would cause a further loss of cells or humoral factors necessary to mediate an immune response, and lead to increased allograft survival times for recipients of F_1 BM.

Studies performed in this laboratory (42,85) suggest that the GVH reaction itself becomes suppressed soon after its onset, and that this self-suppression may be responsible for the receovery of animals from the wasting disease. Grushka and Lapp (42) have reported that transplants of syngeneic bone marrow protected GVH mice from the lethal effects of the GVH reaction while thymus transplants increased the intensity of an existing GVH reaction. The different effects reported by Grushka and Lapp and by Streilein (83) can be explained in terms of the proposed model for GVH induced immunosuppression. The two effects may simply represent different stages of immunosuppression. If syngeneic BM cells are injected before the thymic factor is completely depleted, the BM could act as new targets for GVH reactivity, resulting in a further depletion of thymic factor. Skin allografts made on these animals would have a longer survival time than grafts made on GVH animals not receiving syngeneic BM. If, on the other hand, the F_1 BM is transplanted after the thymic factor has been significantly depleted, no further GVH ac-

tivity may result. Instead, the syngeneic BM would provide the F_1 host with essential hematopoietic cells and reduce the lethal effects of the GVH reaction.

In view of the fact that stimulation of GVH mice with B6 tissue caused a rapid rejection of subsequent B6 skin grafts, attempts were made to detect the presence of specific antibodies following each step in the sensitization process. The use of PVP to augment hemagglutination provided an accurate indication of the presence of low levels of circulating antibodies. Since the PVP assay can detect the presence of both hemagglutinating and complement-fixing antibodies, it is more sensitive than hemolytic or cytotoxic assays. Using this assay it was possible to demonstrate the presence of significant levels of anti B6 antibody in normal mice following each exposure to B6 antigen. It was not possible, however, to detect any such humoral response in GVH mice receiving the same treatment. Similar findings were obtained in the case of animals developing delayed hypersensitivity responses to SRBC. In this case it was impossible to detect a humoral response in GVH mice using both PFC and hemagglutination assays.

In the present experiment no attempt was made to detect macrophage-associated cytophilic antibodies in GVH animals which had rejected skin allografts or developed delayed hypersensitivity responses. The requirement for macrophages in cell-mediated reactions has already been established (7,77,90). Antigen specificity has not been demonstrated in pure macrophage populations and many studies (10,60,67,92,94) suggest that

stimulation and specificity may be conferred upon macrophages by means of cytophilic antibodies or cytotoxic factors. If cytophilic antibodies are necessary for cell-mediated reactions, the ability to stimulate these reactions in GVH mice would indicate that cytophilic antibodies are produced by a different mechanism and/or cell population than those responsible for classical antibodies. This in turn would lead one to suspect that cytophilic antibodies may represent another form of macrophage cytotoxic factors which are produced by sensitized T cells.

Although cell-mediated and humoral immune responses may differ substantially in their mechanisms of action, they nonetheless have many features in common. For example, antigen recognition by sensitized T cells, the interaction of macrophages, and the stimulation of effector cells by the T cells appear to be essential in both types of immune reactions. The fact that it is possible to selectively stimulate a cell-mediated immune response in GVH animals while the humoral response to the same antigens remains suppressed suggests that two distinct mechanisms of T cell activity are involved in the immune response. It is not known whether soluble factors produced by T cells play a helper role in cell-mediated reactions as they do in humoral responses. It is possible that effector cells in cell-mediated reactions may become stimulated by direct contact with sensitized T cells without the need for soluble mediators. In terms of the proposed model for GVH induced immunosuppression, such a mechanism might partially circumvent the suppressive effect of the GVH reaction. In this case the inhibition of T cell

factor release by A cell factor would not totally impair cell-mediated responses. It is also possible that T cell activity may be mediated by soluble factors in both humoral and cell-mediated immune responses, and that the T cell or T cell factor responsible for humoral responses is more susceptible to the suppressive effects of the GVH reaction. In either case, the ability to selectively stimulate cell-mediated immune reactions in GVH mice while the humoral immune response remains completely suppressed suggests that the T cell activities in cell-mediated and humoral immune responses are performed by cells which are functionally different (89). It is not clear, however, whether these cells develop from the same T cell population or represent two distinct cell lines.

Recent evidence suggests that two different types of T cells may be responsible for cell-mediated immunity and helper cell activity in antibody formation. Studies performed by Parish (70,71,72) have demonstrated that humoral and cellular responses are often mutually antagonistic, but these studies failed to delineate the origin of the two types of T cells involved. Chemical modification of SRBC by either periodate oxidation or acetoacetylation resulted in preparations which stimulated lower antibody responses than did normal SRBC, but induced much higher levels of delayed hypersensitivity in the absence of Freund's adjuvant. It appeared therefore that two T cell populations with different binding characteristics may be involved in the two types of reactions.

Several studies suggest that the two T cell functions may be performed by T cells in different stages of maturation. The work of Axelrad (5)

suggested that the development of delayed hypersensitivity and direct PFC to SRBC were dependent upon a common immunocompetent precursor cell. More recently, Elliot et al. (29) have shown that thymus derived rosette forming cells (TRFC) to SRBC do not function as helper cells in the direct PFC response to SRBC. Thymus derived rosette forming cells have been implicated, however, in a number of cell-mediated reactions. Marchalonis et al. (64) have observed increased numbers of TRFC in mice immunized with poly-(A:U) which acts as an adjuvant to enhance delayed hypersensitivity reactions. In addition, Haskill (44) has found increased numbers of TRFC against allogeneic (DBA/2) erythrocytes in the peripheral blood of CBA mice which were rejecting DBA/2 skin grafts.

The work of Elliot et al. (29), Marchalonis et al. (64) and Haskill (44) has shown that helper function in humoral immune responses is a property of non-rosette forming T cells while activity in cell-mediated responses may be a property of TRFC. This would imply a different mode of activity for these 2 cell types. If such is the case, it could explain why it is possible to stimulate cell-mediated responses but not humoral responses in GVH mice. It is quite possible that T cells of the non-rosette forming type are more vulnerable to the destructive and/or suppressive effects of the GVH reaction while rosette forming T cells may be more resistant. This could arise if differentiation brings about surface membrane changes in which histocompatibility antigens become masked by the appearance of the antigenic receptors necessary for

rosette formation. Repeated challenge of GVH mice with a given antigen could stimulate the proliferation of a clone of the more resistant T cells which would be capable of effecting a cell-mediated response. If such were the case, one would expect the resistant TRFC to represent a cell line separate from the non-resistant helper cell. Elliot et al. (29) have suggested that the TRFC may merely represent a more mature stage of the non-rosette forming helper cell. If the non-resistant helper cells represented an earlier stage of the same cell line, a rapid maturation to the resistant stage would be necessary in order to produce enough T cells for a cell-mediated response. This is also possible however, since slowly maturing cells would presumably be destroyed, resulting in the loss of helper function and continued suppression of the humoral immune response.

The proposed model for GVH induced immunosuppression provides an explanation for the suppressive effects of the GVH reaction on humoral immune responses. The mechanisms for cell-mediated reactions are less precisely defined than those for humoral reactions, and it is clear that there are several basic differences between the two mechanisms. Nevertheless, the present experiments have been shown to be in agreement with the proposed model. At present the difference in the suppressive effect of the GVH reaction on the two types of immune responses appears to result in part from the activity of two functionally different T cells. Although much evidence suggests that these T cells represent separate populations, it cannot be definitely stated whether these populations

develop from the same T cell line or from separate lines. As the cell interactions in cell-mediated responses become more clearly defined, it will permit a more precise determination of the factors leading to the selective stimulation of cell-mediated immune responses in GVH immunosuppressed animals.

Application of GVH induced immunosuppression to the normal immune responses.

The GVH reaction represents a unique situation in which the most complex immunological principles and cell interactions are brought together into one reaction. Although GVH reactions are experimental in nature, the underlying principles apply equally to normal immune responses. Characteristics of GVH reactions such as those presented in the present experiments can therefore provide much insight into the normal regulation of cell-mediated and humoral immunity. For instance, adherent cells appear to play a regulatory role in GVH induced immunosuppression, and recent evidence suggests that adherent cells may play a regulatory role in normal immune responses as well (14,50,71,74,78,94).

The parallels between cell interactions in GVH mice and interactions in the normal immune response can be illustrated by the inverse relationship which appears to exist between cell-mediated and humoral immune responses. The present experiments demonstrated that stimulation of delayed hypersensitivity reactions to SRBC in normal mice led to a concomitant decrease in the humoral response of these animals to SRBC. Other

studies (5,53,63) have shown that the stimulation of a humoral response to SRBC causes a significant decrease in the ability of the animal to develop a delayed hypersensitivity response to SRBC. Conversely, Lagrange (52) has found that the removal of antibody from the serum of mice receiving intradermal injections of SRBC in Freund's adjuvant led to delayed hypersensitivity responses which were up to 4 times as great as those produced in normal mice receiving SRBC in Freund's adjuvant. A similarity can be drawn between these reports and the selective stimulation of cell-mediated immune responses observed in GVH immunosuppressed mice. Following the injection of SRBC in Freund's adjuvant in the present experiments, GVH mice produced no detectable levels of antibody to SRBC but developed an almost normal delayed hypersensitivity response to SRBC. Although the delayed hypersensitivity response in GVH mice was not as great as that observed by Lagrange (52) following the removal of serum antibodies, the smaller hypersensitivity response in GVH mice might result from a competition for cells and factors being simultaneously utilized or destroyed by the stronger cell-mediated GVH reaction.

The consistent observation of an inverse relationship between cell-mediated and humoral immune reactions suggests that a common regulatory mechanism may control these two types of immune responses. A basic feature of this mechanism may depend upon the processing of antigen. Numerous studies (48,74,84) have shown that the type of immune response which is produced may depend in part upon the form in which antigen is

presented to antigen reactive T cells. Parish (70,71,72) has demonstrated that chemical modification of SRBC by either periodate oxidation or acetoacetylation resulted in preparations which stimulated lower antibody responses than did normal SRBC, but induced much higher levels of delayed hypersensitivity in the absence of Freund's adjuvant. The work of Stupp et al. (84) has shown that as the molecular size of synthetic antigens is increased they stimulate weaker delayed hypersensitivity reactions but stronger antibody formation. Evidence obtained from the present experiments would suggest that stimulation of a particular immune response by modified antigen may result from the presence of two T cell populations, each with different binding characteristics.

Recent reports (48,50,71,74) suggest that macrophages, by virtue of their ability to process antigen, may play a key role in the regulation of cell-mediated and humoral immune responses. For example, Pearson and Raffel (74) have found that SRBC which have been digested by macrophages become poor immunogens for antibody production but gain the ability to evoke delayed hypersensitivity. It is interesting that some studies (19,24,25) have shown that macrophages treated with mycobacteria are more actively phagocytic and are more active enzymatically than normal macrophages. Consequently, it has been proposed that adjuvant may stimulate delayed hypersensitivity by promoting macrophage activity and the reduction of the sensitizing antigen to a small moiety which is more favorable for cell-mediated reactions. In view of this suggestion, one might wonder whether the increased activity of macrophages

during GVH reactions might favor cell-mediated responses and partly account for the greater ability to stimulate cell-mediated versus humoral responses in such animals. It would be quite possible for activated macrophages to engage in antigen processing which would favor cell-mediated reactions while simultaneously exerting a suppressive effect on humoral reactions.

Macrophages have also been shown to be capable of exerting a regulatory role over other cells by virtue of soluble factors released by the macrophage upon stimulation with antigen (38,39,40,46). Low concentrations of soluble factors produced by peritoneal exudate cells, which are mostly macrophages, have been found to stimulate the primary PFC response of spleen cells to SRBC in vitro (46). High concentrations of these soluble factors are inhibitory. In other studies (37,38,39,40) cultured adherent mouse spleen cell populations, which consist mostly of macrophages, have been found to release effective supernatants which stimulate cell proliferation and potentiate mouse T cell responses to phytohemagglutinin (PHA). Stimulation of the adherent cells with LPS increased the effectiveness of the supernatants but did not cause a mitotic response on the part of the adherent cells. The active factor in these supernatants has been termed lymphocyte activating factor (LAF) (39). The stimulatory effect of LAF would appear to be at variance with other reports of inhibitory effects exerted on T cell activity by macrophages (33,66,78). In such experiments it is important to consider dose-response relationships, since substances which are stimulatory at

low concentrations are frequently inhibitory at higher concentrations. The significance of this point is illustrated by the fact that reports of T cell activation (38,40) are based on observations using culture supernatants which would contain low concentrations of soluble factors. Reports of T cell inhibition by macrophages, on the other hand, come from experiments (33,73,78) requiring cell contact where high local concentrations of macrophage factors would be expected to develop.

By using a biphasic effect of macrophage activity as previously described, one can construct a hypothetical model to explain possible control mechanisms which might regulate cell-mediated and humoral immune responses in both normal and GVH animals (Figure 11). This model presents cell-mediated and humoral immune responses as separate mechanisms sharing a central adherent cell which controls the development of each type of response. I propose that the central adherent cell controls immune responsiveness by the elaboration of a single soluble factor, and that the concentration of this factor determines whether a cell-mediated or humoral response will develop. Although adherent cell populations have been shown to consist primarily of macrophages, other cells may also be present. Because of this fact, the term adherent cells will be used even though these cells are most likely macrophages.

In this model, the initial recognition of antigen is made by the adherent cell. Although immunoglobulin receptors for serologically defined antigens have not been identified on adherent cells, it is possible that products of the Ir region are expressed on the surfaces of these cells (6). The Ir region has been shown to control re-

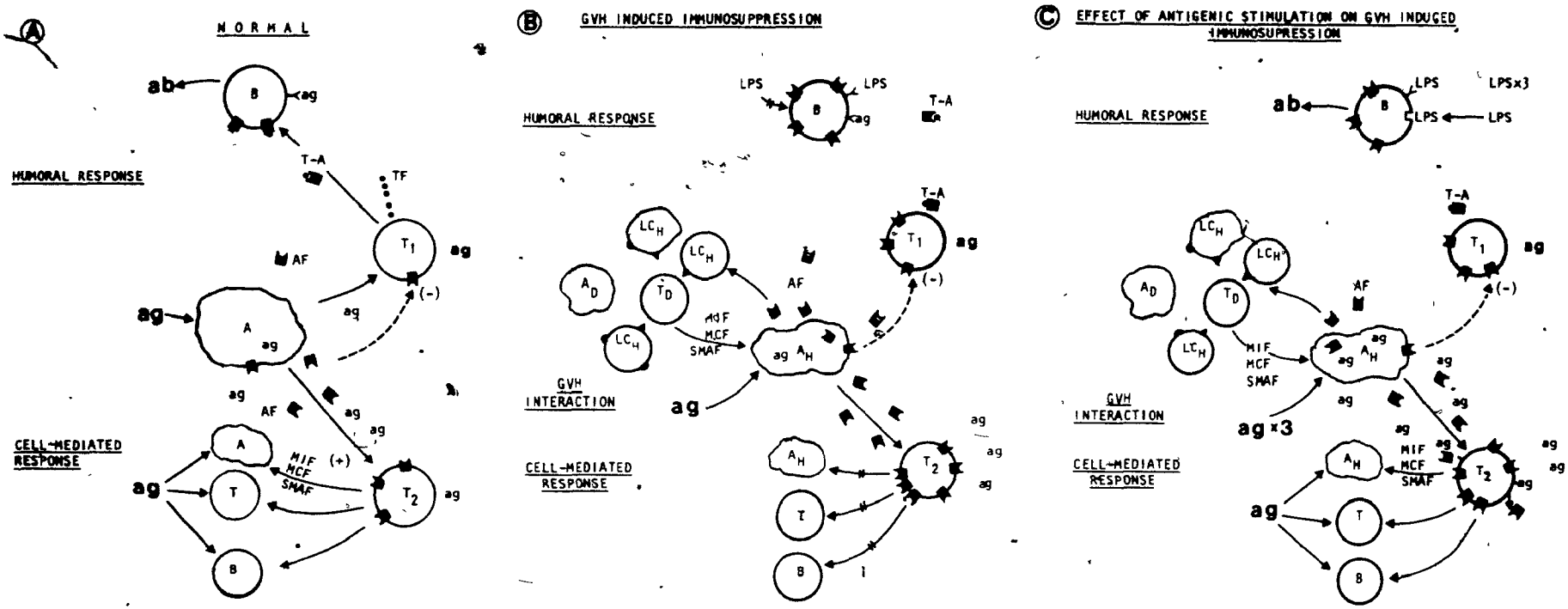

FIGURE 11: Proposed Model for Regulation of Cell-Mediated and Humoral Immune Responses.

a) Normal
b) GVH Induced Immunosuppression
c) Effect of Antigenic Stimulation on GVH Induced Immunosuppression

activity to a number of synthetic proteins and viruses, and products of the Ir region may render adherent cells capable of recognizing lymphocyte defined (LD) antigens. The initial recognition of antigen by an adherent cell would stimulate the A cell to process the antigen and release A cell factor. The extent of antigen processing and the amount of factor released would be determined by the LD components on the antigen and the macrophage activity at the time of stimulation. Adherent cells stimulated by strong LD antigens, adjuvant, or a GVH reaction would process antigen to small units and in so doing release large amounts of A cell factor. Both of these activities would favor the development of a cell-mediated response to the antigen. On the other hand, a lesser stimulus would result in a less complete processing of antigen, with the release of less A cell factor and the consequent development of a humoral response. The A cell factor may merely represent a part of the lysosomal contents such as one of the many enzymes known to be released from macrophages during antigen degradation.

In view of the present experiments, two functionally different T cell populations appear to operate in cell-mediated versus humoral immune responses. I propose that the helper T cell in antibody formation is stimulated by low concentrations of A cell factor but becomes suppressed at higher concentrations of the factor. Conversely, the T cell responsible for cell-mediated reactions is stimulated only by high concentrations of A cell factor. The stimulation of a cell-mediated immune response by a high concentration of A cell factor would consequently lead to a

suppression of the humoral response by suppressing the T cell necessary for a humoral response, as well as by binding to the B effector cell as previously described. On the other hand, the low concentration of A cell factor which would stimulate a humoral response to a given antigen would be insufficient to stimulate a cell-mediated response to that antigen. This would account for the inverse relationship observed between the cell-mediated and humoral responses to a given antigen.

In order for a given antigen-reactive T cell to be activated by A cell factor, the T cell would require a signal from a specific antigen as well as from the A cell factor. Although the initial antigenic stimulation would be a property of adherent cells, antigen specificity to serologically defined antigens would be determined by T cells. T cells involved in humoral immune responses would be preferentially stimulated by larger antigenic determinants while T cells involved in cell-mediated reactions would be stimulated by smaller antigenic determinants. Once a given T cell received these two signals, it would in turn stimulate an effector cell, either by direct contact or by the release of a soluble mediator. In the case of a humoral response the effector cell would be an antibody forming B cell which would require binding of the T-A complex as previously described. In the case of cell-mediated reactions the effector cell could be the same T cell, another T cell, a macrophage, or even a B cell.

Although the GVH reaction is an unnatural situation arising from experimental manipulation, it is nevertheless controlled by the same principles that govern normal immunological responses. The immuno-

suppressive effect of the GVH reaction can therefore be explained in terms of the model which has just been proposed for the regulation of cell-mediated and humoral immune responses. This model provides a unified explanation for the present observations that cell-mediated responses can be selectively stimulated in GVH immunosuppressed animals while the humoral responses to the same antigens remain completely suppressed.

Although antibodies directed against host tissues have been identified in animals undergoing GVH reactions (68,76) these reactions have been shown to be primarily cell-mediated immune responses (3,79). The GVH reaction is most likely initiated by the interaction of donor T cells with host lymphocytes (3,15,16). In addition, donor macrophages may also contribute to the GVH reaction (2). I propose that, following their interaction with host tissue, the donor T cells release factors such as MIF and MCF which recruit and activate both host and donor adherent cells. Donor A cells may also contribute directly to the stimulation of a GVH reaction by responding to lymphocyte defined antigens on host lymphoid cells as described above. The increased activity of macrophages during the GVH reaction would lead to a more complete processing of antigen which would tend to favor cell-mediated reactions.

Once activated by the GVH reaction, donor and host A cells release large amounts of A cell factor which further stimulate the cell-mediated GVH reaction. The high concentrations of A cell factor would cause suppression of the humoral immune response by inhibiting the release of

T cell factor by helper cells and by binding to mitogenic sites on the B cell. Under normal conditions, high concentrations of A cell factor would be expected to stimulate the antigen reactive T cell in the cell-mediated response. During a GVH reaction, however, excessively large amounts of A cell factor would be released by the adherent cells and bind to the T cells. Although the high concentration of A cell factor bound to the T cells would not actively inhibit T cells in the cell-mediated response, it would cause a steric hindrance which would block the binding of antigen to the T cell. The T cell would thus receive a signal from the A cell factor but would not receive the antigenic signal which is also necessary for T cell activation. Consequently, the animal would be unable to mount a cell-mediated response to a single antigenic challenge. Repeated challenges with a given antigen would increase the concentration of processed antigen to a level at which the antigen could overcome the steric hindrance of A cell factor on the T cell. The resultant interaction of antigen with the T cell would provide the second signal necessary for T cell activation and/or proliferation. Once a clone of T cells became activated they would in turn stimulate effector cells which would bring about the development of a cell-mediated immune response. The humoral response would remain suppressed, however, by the active suppression of helper T cells by A cell factor, as well as by the competitive binding of this factor to the antibody forming B cell.

The above model represents one interpretation of the present experimental observations in the light of our current understanding of

normal immune responses. It is an attempt to explain the effects of the GVH reaction in terms of normal immune control mechanisms. This model is not intended as the only mechanism for regulation of the immune system, and it is understood that many processes other than those presented here operate in immune responses. No attempt has been made to provide a detailed discussion of this model in terms of the vast amount of literature on the subject of immune regulation. Although many reports may seem at variance with this model, it is believed that the model presents the basic concepts of immune regulation and relates them in a form which is in agreement with the majority of information available. Clearly, much research must still be done to further delineate the regulatory mechanisms controlling normal immune responses as well as those leading to GVH induced immunosuppression.

Application of the principles of selective stimulation of cell-mediated immune responses.

The ability to selectively stimulate a cell-mediated immune response in GVH mice can provide valuable information leading to the selective stimulation of cell-mediated responses in normal animals. Such control of immune responses may have important applications in the treatment of neoplastic growths where it has been suggested that antibody against tumor specific antigens may protect tumor cells from immunological rejection. Experiments performed by us have shown that the selective stimulation of cell-mediated responses in GVH mice can, in fact, protect these mice from tumor growth (57).

The injection of live Sarcoma I cells into normal and GVH mice resulted in a rapid growth of the tumor. Sensitization of GVH mice with dead, lyophilized Sarcoma cells 7 days prior to the injection of live tumor cells caused a marked decrease in the rate of tumor growth. A similar sensitization of normal mice with lyophilized tumor cells did not significantly alter the rate of tumor growth. The results of the present experiments would suggest that a cell-mediated immune response was selectively stimulated by the injection of dead Sarcoma cells and that this cell-mediated response had an inhibitory, if not cytotoxic, effect following the injection of live tumor cells.

Further studies are necessary to delineate the mechanisms regulating the interaction of cell-mediated and humoral immune responses in both normal and GVH animals. A clearer understanding of these mechanisms could have a wide range of clinical applications such as the selective stimulation of a cell-mediated response to cause tumor rejection or the selective suppression of a humoral response to prevent allergic reactions. Although the applications of experiments such as the present study are not always immediately evident, their value lies in presenting a clearer understanding of basic immunological principles which can then be applied to clinical situations.

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SUMMARY

1. Suppression of the humoral immune response to SRBC in CAF_1 and $B6AF_1$ mice was shown to depend upon the strength of the GVH reaction and its duration at the time of sensitization to SRBC. Whereas immunosuppression was preceded by an increased responsiveness in CAF_1 GVH mice, suppression of the humoral immune response to SRBC in $B6AF_1$ mice declined steadily from the first day after induction of the GVH reaction. In both strain combinations the animals were totally unresponsive to SRBC by 7 days post GVH induction and remained unresponsive through day 100.

2. Adrenalectomy and castration of CAF_1 mice prior to the induction of a GVH reaction did not reduce the immunosuppressive effect of the GVH reaction. This suggests that immunosuppression results from immunological factors rather than from a general stress response.

3. Multiple challenges of CAF_1 GVH mice with B6 allotransplantation antigen caused a rapid rejection of subsequent B6 skin grafts, but failed to stimulate the production of any detectable antibodies to B6 antigens.

4. Sensitization of GVH mice with SRBC in Freund's complete adjuvant stimulated a delayed hypersensitivity response to SRBC although no humoral response to SRBC could be detected, even after three challenges with SRBC.

5. Appropriate stimulation of GVH mice was therefore capable of eliciting cell-mediated reactions to two different types of antigens while the humoral immune responses to the same antigens remained completely suppressed.

6. Although a single challenge with the thymus-independent antigen LPS did not elicit a humoral response in GVH mice, a second and third challenge with LPS resulted in the production of a significant number of LPS-specific PFC and a high titre of anti-LPS hemagglutinating antibodies.

7. The experimental results are discussed in terms of a proposed model for the regulation of normal immune responses.

Note: The above summary outlines the original work contained in this thesis.

DEFINITIONS

Adjuvant: A substance which when mixed with an antigen enhances its antigenicity.

Alloantigen (isoantigen): An antigen which incites the formation of antibodies in genetically dissimilar members of the same species.

Allogeneic: Pertaining to genetically dissimilar individuals of the same species.

Allograft (homograft): A graft derived from an allogeneic donor.

Autograft: A graft derived from the same animal to which it is transplanted.

Cell-mediated Immune Response: An immune response such as graft rejection or delayed hypersensitivity. These reactions are characterized by direct cytotoxic activity of immunocompetent cells and can only be transferred to another animal with cells from a sensitized animal.

Clone: A population of cells derived from a single cell by asexual division. All cells within the population are therefore of the same genetic constitution.

Delayed sensitivity: A specific sensitive state characterized by a delay of many hours in onset time and course of reaction. It is transferable with cells but not with serum.

Freund's adjuvant: (a) Complete = Freund's water-in-oil emulsion of mineral oil, plant waxes, and killed tubercle bacilli used to incorporate with antigen to stimulate antibody production; (b) incomplete = Freund's mixture without tubercle bacilli.

Graft vs host reaction (GVH): An immune response produced when immunocompetent cells are grafted to an incompatible host and react against the host tissue.

Hemagglutinin: An antibody which reacts with a surface antigen-determinant(s) on red cells to cause agglutination of the red cells.

Histocompatibility antigens(transplantation antigens): Antigens coded for by "histocompatibility genes" which determine the compatibility of grafted tissues and organs.

Humoral immune response: An immune response resulting in the formation of a soluble antibody which is released into the body fluids where it is capable of reacting with a specific antigen. Reactions of this type can be transferred to an unsensitized animal with serum from a sensitized animal.

Second-set graft rejection: Accelerated rejection of a second graft due to immunity developed to a primary graft.

Syngeneic: Pertaining to genetically identical or near-identical animals such as identical twins or highly inbred animals.

Xenogeneic (heterologous): Pertaining to individuals of different species.

Reference: Weiser et al., #240, Chapter 1.

Abbreviations used in Text

AF	A cell factor
B6	C57BL/6
$B6AF_1$	C57BL/6 X A F_1
BM	Bone Marrow
BSS	Balanced Salt Solution
CAF_1	CBA X A F_1
CFA	Complete Freund's Adjuvant
DH	Delayed Hypersensitivity
F_1-LPS	F_1 RBC coated with LPS
GVH	Graft-versus-host reaction
H	Histocompatibility
KC	Kidney cells
LPS	Lipopolysaccharide
MST	Median survival time
PBS	Phosphate buffered saline
PFC	Plaque forming cells
PVP	Polyvinylpyrrolidone
SE	Standard error
SRBC	Sheep red blood cells
SRBC-LPS	SRBC coated with LPS
T-A	TF-AF complex
TF	T cell factor