B CELL HETEROGENEITY: DIFFERENTIAL MODULATION OF MURINE B-SUBPOPULATIONS BY ALLOREACTIVE T LYMPHOCYTES

by

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STATEMENT OF PROBLEM

The main goal of the work to be presented in this dissertation is to broaden our understanding of B-cell functional heterogeneity. In order to understand the reason for the differences in the type of stimuli required for their activation we have adopted a system in which the importance of antigen (various forms of TNP-carriers), antigen-specific, and antigen-nonspecific regulatory signals in triggering (or preventing) B cell differentiation could be evaluated.

The model system used, "allogeneic effects", is a well characterized system in which both enhancing (positive) and suppressor (negative) effector T cells are triggered following recognition of alloantigens on stimulator B cells. Once stimulated, they are capable of interacting with B cells and modulating their antibody responses irrespective of the antigen used. The main questions which will be approached are:

1) Are B cell subpopulations similarly or differently modulated by allogeneic effector cells _in vitro_ or _in vivo_?

2) Are primary (unprimed) and secondary (memory) B subsets similarly affected by allogeneic cells?

3) Using this model what can we learn about the physiological state of B cells belonging to different subpopulations and the ontogenic relationship between them?

A separate portion of this dissertation will deal with the cross reactivity among IgD surface receptors on B cells from 3 different mammalian species (mouse, rat and human) and the relevance of our findings to the question of how this ubiquitous B cell receptor has evolved.

INTRODUCTION AND LITERATURE REVIEW

B lymphocytes: a model for studies of cellular differentiation

The lymphoid system offers an ideal model for studies of cellular differentiation since unlike most other tissues which differentiate mainly during embryogenesis, the lymphoid pool is continuously replenished throughout the life span of the individual from a primordial hematopoietic stem cell pool which in mammals resides in the bone marrow. This pool gives rise to precursors of the erythrocyte, monocyte/polymorph and the lymphocyte lineages. The prolymphocytes then, through multiple stages of differentiation give rise to either T or B lymphocyte populations which are capable of recognizing "self" and/or nonself antigenic determinants by highly specialized surface receptors. Recognition of antigen leads these cells to execute a variety of immunological functions. Their differentiation occurs via sequential expression of new genes which result in the appearance (and/or disappearance) of surface determinants or "markers" some of which are directly involved in the functional capability of the mature T and B cells. In addition, B and T lymphocytes are capable of "communicating" among themselves via soluble factors or cell-cell interactions forming a so called "immunological network" which maintains the delicate balance among the various elements of the system. Aberations of this balance (which usually occur more frequently in neonates or in aged individuals) result in immunological insufficiency or hyperreactivity (e.g. autoimmune diseases).

If one focuses on any single differentiation step in the developmental pathway of T or B lymphocytes several questions come to mind: 1) Does this step reflect the result of an already prescribed program which activates new sets of genes which in turn allow the expression of new functional properties? 2) What is the nature of the inducing agent which physiologically delivers the signal which puts the program into effect? 3) Where does this signal come from, how is it delivered to cells and how is its message transmitted from the cell surface to the nucleus? And finally: 4) Can a single cell give rise to only one or more than one type of progeny? If the latter, does this choice depend upon the nature of the inducing agents?

At least some of these questions may be approached if one is able to identify and to isolate lymphocytes at any particular differentiation stage through some unique phenotypic markers and has developed an in vitro system in which differentiation can proceed.

The main theme of this thesis is the B-lymphocyte lineage. A large body of data has been accumulated during the last several years concerning the gene rearrangements that lead to the surface expression and secretion of antibodies by mature B cells (1-4). But what are the signals which drive pre-B cells into mature, antigen sensitive B lymphocytes? And what factors determine whether a stimulated primary B cell will differentiate into a plasma cell or instead, give rise to a memory cell which may circulate in the body for many years and subsequently be driven to antibody production by a second encounter with the relevant antigen? Our present knowledge of B cell ontogeny emerges from developments in four major areas of immunological research:

(1) The development of *in vitro* systems which support differentiation of immature B cells and sequential acquisition of surface markers. For example, Hoffman et al. found that in a Komura-Boyse induction assay, interleukin 1 (Il-1), a factor produced by LPS activated macrophages, induces early B cell markers (surface Ig, Ia) as well as complement receptor (CR) (5-7), which ontogenically is a late B cell marker (8). Others were able to obtain Ig expression and surface isotype switch in pre B cells cultured *in vitro* with the B cell mitogen LPS (9-11).

(2) Studies of B cell lines which may represent various differentiation stages. Some of these lines have arisen spontaneously in animals (12-13), others have been produced in different labs either by cell hybridization techniques (14-15) or by transformation of immature B cells by Abelson murine leukemia virus (16-18). Some of these lines are inducible *in vitro* and will be most useful for biological and biochemical analysis of B cell differentiation and activation.

(3) Studies of mutant animal strains carrying genetic defects which effect mainly or selectively the B cell lineage (19-22). The most characterized mutant strain is the CBA/N. These mice carry an x-linked immune defect which is associated with a deficiency of a mature or late developing subpopulation(s) of B cells. Phenotypically, their B cells resemble neonatal cells (23-24). In addition, they have deficient responses to certain T-independent antigens (TI) such as TNP-Ficoll and DNP-Dextran which have been termed TI-2 antigens, but do respond to other TI antigens which have been categorized as TI-1 antigens (e.g. TNP-LPS, TNP-*Brucella abortus*) (21-22, 25-26).

This latter group of antigens is capable of stimulating a larger array of B cell clones, belonging to both early and late development stages (27). CBA/N mice also respond to T dependent antigens although only to high doses (28-29). Heterozygous F1 males which carry the CBA/N x-chromosome also exhibit the immune defect. Thus by immunizing (CBA/N x BALB/c) F1 male mice with BALB/c spleen cells, it was possible to obtain an antiserum which reacts with a component of mature B cells. This component was termed Lyb-3. Another antiserum was raised in C57BL/6 mice against DBA/2 splenocytes and was subsequently absorbed extensively with (CBA/N x DBA/2) F1 male lymphocytes. The resulting antiserum recognized a marker on mature B cell populations which was termed Lyb-5 (30-31). Another B cell surface marker LyB-6 was recently detected and isolated using antiserum from CBA/N mice immunized with CBA/J (nondefective) cells. This marker reportedly is present on the majority of normal splenic B lymphocytes (32).

Whether or not these B cell markers are directly involved in triggering of those B cells which bear them is not clear yet, although recently it was demonstrated that Lyb-5 negative B cells fail to be triggered by antigen-presenting accessory cells regardless of the antigen being presented (33-34).

(4) The development of <u>in vitro</u> cultures suitable for stimulation of unprimed or primed murine B cells (35-36) allowed dissection of the antibody response and identification of the cellular interactions and signals which are involved in normal (or abortive) stimulation of B lymphocytes. It was found that antibody responses, particularly of

the IgG isotypes proceeds usually through a proliferative phase followed by differentiation of the activated B cells into antibody producing plasma cells (37-40).

The signals which initiate B cell proliferation may be received via their surface Ig receptors following interaction with the relevant antigen. (or with anti-Ig antisera) (41), or via mitogen receptors such as LPS (42). These signals, however, may not be sufficient to drive the stimulated B cells into antibody production. Various B populations are also sensitive to signals delivered by T cells and/or macrophages (43-45). At least some of these signals are delivered in the form of soluble factors and may have a decisive role during either the early or late phases of the response. Antigen specific factors which are produced by helper T cells, and a macrophage product-interleukin I, are both examples of factors which initiate B-cell division, in concert with the specific antigen (44, 46). Other T cell signals (non antigen specific), such as T cell replacing factor (TRF) act at a later stage of the response, converting the proliferating B cells into plasma cells (43, 47-48). In certain antigenic systems (e.g. anti-phosphorylcholin response) there is ample evidence for the role of yet another type of helper T cell (termed TH_2) which selectively activate certain B cell clones which produce antibody of a particular idiotype (49-51). It is believed (but not totally proven) that they exert their effect during the early phase of the response.

The two (or three) signal hypothesis and the importance of antigen-Ig receptor interaction during B cell activation have been challenged recently by Cammisuli et al. (52-53). These investigators

devised an *in vitro* system in which memory B cells (to the lac hapten) were stimulated to produce lac-specific antibodies not by their relevant antigen, but instead by a surface "labeling reagent-sandwich" which was able to focus T cell help onto the primed B cells. Thus B cells were first treated with benzenearsonate (ars)-coupled to either anti-H-2, or anti-Ia antibodies, followed by a conjugate of anti-ars antibody with KLH. The culturing of these KLH-labeled B cells with KLH primed T cells (in the absence of antigen) led to a pronounced production of lac-specific IgG PFC. They concluded that the T cells bear the most central role in B cell activation and once a specific T cell is in close proximity to a memory B precursor it conveys both the differentiative and the proliferative signal to it. However intriguing, one must be cautious in interpreting the results of this rather artifical system in which B cell surface perturbations, which may include some aggregations of the surface Ig receptors, might have happened during the multi stage labeling procedure, thereby rendering the cells sensitive to signals from the added T cells. Furthermore, the same system did not support differentiation of primary precursors and led to activation of only a portion of the secondary precursors (52-53). In conclusion, it might be safely stated that B cell activation can occur via more than one mechanism and depends on the system used. Various surface receptors may play a larger or a smaller role in transducing the signals across the cytoplasmic membrane. Which of these receptors are the principal signal acceptors *in vivo* is also an open question at this stage. However, knowing the highly specific nature of the humoral responses one would expect the surface Ig

receptors to play a central role at least in the early stages of an immune response.

B-cell heterogeneity

B lymphocytes have evolved with an enormous variety of immunoglobulin receptors capable of recognizing a very large array of antigenic specificities. In addition to the heterogeneity at the level of the antigen receptors, considerable evidence has accumulated in recent years for the existence of "functional heterogeneity" among B cells. In particular, there appear to be separate populations of B cells, one responsive to T dependent (TD) antigens (B2 cells) and another (B1) responsive to T-independent (TI) antigens. As will be clarified later, the designation TD and TI responsive B cells is not completely accurate. However, this general nomenclature has been retained for simplicity. Following the *in vivo* studies by Playfair and Purves (54) who were the first to propose two B subpopulations, this concept was examined and substantiated in *in vitro* primary responses. Jennings and Rittenberg reported that stimulation of unprimed murine spleen cells with TD and TI forms of the TNP hapten (TNP-KLH and TNP-T4 respectively) simultaneously, resulted in a primary response which was either additive (i.e. equalled the sum of the individual *in vitro* responses to either antigen alone) or occasionally, synergistic (55). Similarly, additive responses between TD and TI forms of phosphorylcholine (PC) were obtained by Quintans and Cosenza (56). These investigators, using limiting dilution and burst size analyses also found that while the frequency of TI responding PC-specific precursors

exceeded the frequency of the TD responding precursors, the burst size (or the number of divisions following antigen stimulation) of the individual precursors was 4-5 times greater than that of the B1 precursors. These in vitro studies have been confirmed in vivo using the splenic focus technique which is an in vivo limiting dilution assay. Fung and Kohler (57) reported that immunization of mice with TD and TI forms of PC resulted in a combined PC-specific precursor frequency which was "super" additive (i.e. synergistic). While they interpreted this latter observation to indicate the presence of a third population, it is equally possible that the presence of excess helper cells in the system leads to release of some antigen non specific lymphokines which cause additional B1 precursors to differentiate to antigen sensitivity (Steve Kaattari, unpublished observations and my own results, see below). In addition, they found that the TD induced response was relatively resistant to tolerogen and anti-idiotype suppression, whereas the TI response was extremely sensitive to both manipulations (57). Both in vitro and in vivo studies have also indicated that under certain conditions TNP-specific B1 and B2 primary cells may differ in their susceptibility to tolerance induction (58) and to certain drugs such as azathioprine (59) and cyclosporin A (60). The relative dependence of TD and TI responses on macrophages (MØ) may also differ. Early studies suggested that greater numbers of MØ were required for optimal TD responses than for optimal TI responses (61, 62). However the interpretation of these studies is complicated since the number of MØ required for activation of the helper T cells (TH), which are required for a successful TD

response may be different and possibly higher than the number of macrophages required by B cells in either a TD or a TI response. Recent studies have shown that both TD and TI-2 responsive B cells have absolute requirement for macrophages and can be triggered by antigen-pulsed macrophages (33, 63). TI-1 responses (TNP-Ba) on the other hand, can be initiated either by antigen-pulsed MØ or by soluble antigen in the relative absence of macrophages in the culture (33), although this latter point could merely mean that TI-1 antigens utilize only a very small number, perhaps a subpopulation of macrophages.

The CBA/N model (discussed above) has provided evidence for additional heterogeneity among B cells responsive to TI antigens. It has been suggested that a less mature B1 population is capable of responding to TI antigens such as TNP-LPS and TNP-Brucella abortus which were termed TI-1 antigens, while a more mature B population (which is deficient in the CBA/N mouse) responds to TI-2 antigens such as TNP-Ficoll, DNP-Dextran and other polysaccharides (21-22, 25-26) and a third population to TD antigens. This would suggest a "three subpopulations" model which is not compatible with all of the available data. For example in limiting dilution "addition experiments" described by Lewis and Goodman (64) it was found that the TNP-LPS response (TI-1) was additive with a TI-2 (DNP-Dextran) response but not with a TD antigen (TNP-KLH) suggesting that TI-1 type antigens may actually stimulate B2 cells but in a T independent fashion. This

alternative interpretation of the CBA/N model has recently been supported and expanded in studies of memory subpopulations conducted by Tittle and Rittenberg (65).

TNP specific memory cells which give rise to IgG PFC after in vivo or in vitro challenge with the TNP hapten, can be elicited in mice by hyperimmunization with a TD form of this hapten (TNP-KLH) (66-67). This memory pool contains both TD and TI responding Bγ precursors which were shown to belong to distinct subpopulations as judged not only by the additivity of the in vitro secondary responses to TD and TI conjugates of TNP in conventional and limiting dilution cultures, but also by the ability to kill selectively either population if stimulated with the relevant antigenic form in the presence of a thymidine analogue-bromo uridine deoxyribose (BUdR) and subsequently exposed to light (68). These studies have also demonstrated that killing of TNP-Ba or TNP-LPS (TI-1)-responding B cells eliminated all subsequent responses to TD or TI-2 type conjugates. More importantly, although neither TD or TI antigen given alone prior to BUdR and light treatment could block a subsequent response to TI-1 antigenic challenge, the combination of TD plus TI-2 prior to BUdR treatment completely blocked responses to TI-1 (TNP-Ba or TNP-LPS) antigens. These results suggest that at least in the memory pool, TI-1 antigens probably stimulate both B1γ and B2γ populations to proliferation and antibody secretion and there is no need to invoke a putative third population (69). The selective killing of B-subpopulations while supplying a very strong evidence for their existence in the memory pool has not been applicable in studies of primary B-subsets; selective killing

has not been achieved (Quintans and Cosenza, G. Lewis, personal communication, and Jennings and Rittenberg, unpublished.) Unlike B1γ and B2γ memory precursors, their primary B1μ and B2μ counterparts can be driven into proliferation (but not antibody production) unselectively by all forms of antigen, TD, TI-1 or TI-2 (70). These findings suggested that primary and secondary B subsets differ not only in the class of antibodies produced and the kinetics of the response but also have different activation requirements, as suggested recently by others as well (71). For example, primary B2 cells appear to be differentially sensitive to major histocompatiblity complex (MHC) restrictions in comparison to memory B2 cells (72) and primary and secondary populations also exhibit differences in adherence properties (73), in migratory characteristics (74), various surface receptors and markers (75-77) and in the functional valence of their cell surface antigen receptors (78).

Two of the key questions concerning B subpopulations are: 1) Do they express the same set of Ig-V-genes (or bear the same idiotypes)? and 2) What is the ontological relationship between the functional subsets? Do they represent completely independent lineages which diverse at a very early stage of stem cell differentiation? Or alternatively, do they represent different developmental stages along the same lineage. In approaching these questions one should consider separately primary subsets which develop in the neonates (or emerge from the adult bone marrow) and compare them to memory B subsets which are generated during hyperimmunization with a T-dependent antigen.

Neither of the above questions has been fully resolved yet. Using the hapten inhibition profiles to determine the heterogeneity of the memory TNP-specific TD and TI PFC responses, different profiles were obtained with the TD response being more heterogeneous and containing more clones of higher avidity [Tittle and Rittenberg (79)]. Similarly, in the dextran system, it was shown recently (80) that in a primary response, Dextran-hemocyanin which is a TD antigen stimulates three times more precursors than dextran itself (TI antigen). Furthermore, it was found that while all of the TI responsive clones recognize the α1-3 linkage in the dextran polymer, about 10% of the TD responding precursors were specific for the α1-6 linkage, suggesting that some V genes are used selectively by the TD dextran-specific clones, or that the α1-6 linkage become immunogenic only after conjugation of Dextran to a protein carrier. These studies also could not answer the question of whether the B1- specificities are included in the B2 repertoire or whether they use nonoverlapping V genes.

The ontogenic relationship between B1 and B2 subsets is no less controversial. In earlier studies on the ontogeny of TD and TI responses it was found that normal neonatal animals do not respond to TD antigens while able to respond to TI antigens and mitogens (81-84). Based on these findings and also on the fact that adult B1 cells showed high, neonatal-like sensitivity to tolerance induction it was suggested by Cambier et al. that B1 precede B2 cells in a common lineage (58). A more careful ontogenic study revealed that neonatal spleen cells can respond to TD antigens if suppressor T cells are first removed and adult helper T cells are added (85-87), thus the

above studies cannot be used as evidence for the B1→B2 model. The CBA/N mouse, with its immature B cells which respond to TD antigens but not to TI-2 antigens would lend support to the alternative hypothesis that B2 preceeds B1 in a common lineage (88). This view was originally proposed by Andersson and Blomgren (89) who observed that although polyvinyl pyrolidone (PVP) is a TI antigen in adult mice, it behaved like a TD antigen when used for stimulation of neonatal spleen cells in vivo (90).

The possibility that B1 and B2 primary populations develop independently of each other along separate lineages has also been suggested by Kincade (91) who made the observation that CBA/N spleens are devoid of B cells able to form colonies in soft agar (which include both B1 and B2 in normal mice). Furthermore, in recent studies of in vitro cultures of Lyb5 positive and Lyb5 negative B cells, it was demonstrated that the TD-responsive population in CBA/N mice also lack a mature subset ($Lyb5^{+}B2?$) resulting in their inadequate stimulation in vitro, in particular by antigen-pulsed macrophages (34, 92). This view was further developed recently by Marshall-Clarke and Playfair (93) who suggested that B1 and B2 could represent separate lineages whose phylogenetic origins are rooted in the need of the primitive B cell systems to deal with various aspects of bacterial infections. Thus the B1 lineage developed in association with immunity to bacterial capsules and flagella (which can elicit TI responses) while the B2 lineage emerged from the need to deal with bacterial exotoxins since proteins are mostly TD antigens.

The ontogenic relationship between the memory subpopulations (B1γ and B2γ) and between them and their primary counterparts B1μ and B2μ are even less understood. It is true that successful generation of memory IgG precursors of both subpopulations depends on the use of a TD antigen for immunization (67, 94-95). However, the possibility that the TD antigen may, after some processing *in vivo*, stimulate primary B1μ clones to give rise to memory B1γ cells (but not to primary PFC) has not been disproven. One possible approach is to find a way to immunize animals under conditions which favor suppression of the TD (but not TI) precursors. If memory B1γ develops normally even though B2γ do not, it would suggest separate lineages. If, on the other hand it is found that the development of all memory clones is impared under such conditions, a common lineage with a primary B2 precursor giving rise to both B2γ and B1γ memory populations would be a more logical conclusion. This approach has been applied in our laboratory and the results are presented in manuscript #3 of this thesis.

Several investigators have reported that sedimentation velocity can be used to separate B cells into size populations which appear to be in different developmental stages of maturity (96-98). Shortman et al. found that spleens of adult mice contain two distinct categories of Ig^+ lymphocytes that could be physically separated from one another. One category had the physical properties of typical mature small B cells, which could be stimulated *in vitro* with antigens and give rise to PFC within 3-4 days in culture. This population was activated into cell cycle by specific but not by unrelated antigens (99). The second category of B cells, larger in size than the first

one could only be stimulated in an in vivo "adoptive" transfer assay giving rise to antigen specific (NIP-POL in their system) PFC response only after 8-9 days. Shortman et al. (100) also found that this latter category of cells could be activated into cell cycle (but not into antibody production) by the injection of a variety of unrelated antigens (such as horse erythrocytes or PPD) 3-4 days before the specific antigen. The subsequent specific response to antigen under such conditions was markedly enhanced. The first category of cells was termed "direct-PFC-progenitors" while the second category of apparently less mature cells was called "pre-progenitors". It was postulated that the "preprogenitor" population gives rise to the direct progenitors which are smaller in size and can be stimulated in vivo or in vitro by the specific antigen (101). The nature of the non-specific stimulus activating the pre-progenitor was not fully resolved although it was found to depend on the presence of mature macrophages but not T cells (101). The presence of preprogenitors in the memory pool was also indicated although no physical separation was possible. But the same protocol of non-specific stimulus followed by the specific antigen did elicit heightened IgM and IgG PFC responses in vivo (102-103). In a recent publication it was suggested that primary preprogenitors reside mainly in the $IgM^{+}IgD^{-}$ cell pool and to a lesser extent in the $IgM^{-}IgD^{-}$ pool (104). Since only T independent antigen was used in their system it is not clear whether preprogenitors are unique to the B1 lineage or reside in both B1 and B2 cell pools. Some of the data presented in this thesis support the concept of preprogenitors especially in the B1 pool and suggest that certain antigen non-specific T

cell signals may drive primary or secondary B1 preprogenitors into antigen-responsive "direct progenitors" under *in vitro* culture conditions.

Graft-versus-host reaction (GVHR) and in vitro allogeneic effects

Graft-versus-host reaction (GVHR) occurs when immunocompetent lymphoid cells are transferred from a donor to an allogeneic host. Donor lymphocytes recognize alloantigens on host cell membranes and are stimulated to proliferate. If the host has a reduced immune competency (neonatal mouse or immunosuppressed recipient), then donor cells can invade and damage host tissues causing a runting syndrome, termed graft-versus-host disease (GVHD). This condition is manifested by weight and hair loss and diarrhea. In severe cases it results in death of the recipient. Clinically GVHD frequently follows bone marrow transplantation (105).

The GVHR can be divided into a proliferative phase and an effector phase.

In the mouse, the main stimulus for the "proliferative phase" of the GVHR is provided by an incompatibility in the I-A subregion of the H-2 region which is the mouse major histocompatibility complex (MHC). K-or D-region differences induce much weaker GVHR, whereas I-C subregion differences require the preimmunization of the donor to produce GVHR. The I-B and I-J subregions have not been shown to induce GVHR. In GVH disease however, K, I-A or D differences induce about the same degree of mortality (106-109).

During the course of GVH disease there is a biphasic change in the immunologic status of the host. The initial stage is marked by a prominent stimulation of host B cells by donor T cells. This phenomenon was called the "allogeneic effect" because it was first described in guinea pigs given allogeneic lymphocytes (110-112).

Similar in vivo induced enhancing allogeneic effects have been subsequently described in mice, hamsters and rats (113-116). Although this effect was first reported in primed animals, it may also provoke primary sensitivity against antigens that are either non immunogenic or tolerogenic (e.g. TNP-D-GL) (113). In unprimed animals the allogeneic effect may increase IgM responses to DNP-coupled to polysaccharide or protein backbones and trigger diverse B cell clones since isoelectric focusing of immune sera from GVH mice revealed considerable antibody heterogeneity (117). Also, a high frequency of autoantibodies with reactivities against autologous erythrocytes, epidermal cells, and other "self antigens" (118), as well as anti-DNA antibodies (119) has been detected in hamsters undergoing systemic GVH disease. The allogeneic effect can also overcome the requirement for carrier specific T cell to synthetic polypeptides in so called non-responder mice (120). These findings led to the hypothesis that donor lymphocytes stimulated by host alloantigens may produce soluble mediator(s) that stimulate host B cells and in some cases host T cells (111, 121). The allogeneic effect was found to be contingent on the presence of theta-positive allogeneic donor cells (namely T cells) and may operate at least in one system when mitomycin C treated donor allogeneic lymphocytes were used (115).

All of the above phenomena take place shortly after administration of the allogeneic cells plus antigen. As the GVH disease progresses there are increasing indications of reduced immunocompetence. Significant depression may occur as early as 4-7 days after initiation of the GVH. The spleen of the recipient animals contains T cells of donor origin which are capable of abrogating an in vitro primary or secondary antibody response to sheep erythrocytes (122). In general, during the course of a GVHR, the positive allogeneic effect is a transient phenomenon which is followed by a marked and prolonged immunosuppression affecting humoral immunity and to various degrees cellular immunity (123-124). However, there is an apparent dichotomy in the ability of the host to recover some competence since multiple immunization of the suppressed animals evoked cell mediated immunity but did not reproduce humoral immunity to an antigen such as sheep erythrocytes (124). It was suggested that the GVH process interferes with host T-B interactions through production of either an inhibitory cell or cell product, but does not modify T-cell functions directly. It is also possible that host B cells are destroyed or suppressed preferentially when compared to host T cells, or that the recovery of T cell functions occurs most readily. Several papers have recently suggested that not all B cells are equally suppressed (or killed) by the stimulated donor T cells, in that TI responses to mitogen (LPS) or TI-antigen (PVP) are usually less suppressed and often enhanced while the TD responses are markedly suppressed (125-127 and our own results).

In general then it can be stated that animals undergoing GVH reaction may demonstrate both heightened and suppressed immunoreactivities

depending on donor cell dosage and the time of antigen administration after GVH initiation (125) and possibly, the type of antigen used for immunization. The nature of the cells which mediate these disparate immunoregulatory effects have been studied by several laboratories. It was found that both the enhancing and the suppressing effects were eliminated when GVH spleen cells were treated with anti Thy 1.2 serum and complement and must therefore, be attributed to T lymphocytes. It was further demonstrated that the augmenting and suppressing effects are mediated by T subpopulations which can be distinguished by various physical means such as their sensitivity to irradiation, their Ly phenotype and their frequencies in the GVH spleens (21-28).

In recent years it was found that during the course of an in vitro mixed lymphocyte reaction (MLR) various types of effector mechanisms are generated which closely resemble those generated during the in vivo induced graft-versus host reaction. Both the enhancing and inhibitory influences (designated positive and negative allogeneic effects respectively) have been reproduced in vitro with cells from mixed lymphocyte cultures (129-134). In analogy with antigen and mitogen-induced helper and suppressor systems it was found that the cells that mediate positive and negative allogeneic effects belong to distinct subsets of T cells. The activity of allohelper is irradiation and mitomycin-resistant while that of allosuppressors is sensitive to both treatments (135-136). Furthermore, activated allohelpers and allosuppressors are titrated out independently of each other under limiting dilution conditions (133, 137), and are separable on Ficoll-hypaque gradient (138). The two effector populations also differ in

their mode of action. Katz et al. and others have found that allogeneic helpers can be replaced by a soluble factor found in the supernatant of mixed-lymphocyte cultures (139-140). This factor is composed of two components one of which is an Ia product which probably originates from the surface of the stimulator B cells (139-141). The allosuppressors, on the other hand act by direct suppression of the responding B cells (137, 142 and our own results) and cannot be replaced by soluble factors. This is in contrast with other suppressor systems in which T cells were found to be the immediate targets of antigen-induced suppressor T cells and the suppressive effects could be manifested by soluble antigen specific suppressor factors (143-145).

Thus, the in vivo induced graft-versus-host reaction or the in vitro, MLC-stimulated lymphocytes produce non antigen specific regulatory effector cells which recognize and act directly on histoincompatible B cells. The issues in question here are: 1) Are all B cell subsets in the primary or secondary pools equally sensitive to allogeneic effects; and 2) Is any particular stage along the differentiation pathway of a B cell lineage more sensitive and/or refractory to the augmenting or inhibitory allogeneic effects? These questions are addressed in manuscripts 2 and 3 of this thesis.

Surface IgD-function, Structure and Evolutionary View

In adult mammals, mature B lymphocytes are constantly being generated from stem cells located in the bone marrow (146). The stem cell differentiates into a pre-B cell that expresses cytoplasmic μ-chains

but not light (L) chains (147-148). Such cells have been found in livers from 11-12 day mouse, 7 week human and 21 day rabbit fetuses (149-151). The pre-B cells then undergo additional changes and become small B lymphocytes which synthesize light chains as well as μ-chains. These nondividing B lymphocytes synthesize monomeric IgM molecules (M_2L_2) that are placed on the plasma membrane to act as antigen receptors (152). The immature μ-positive B cells migrate to the spleen and lymph nodes and divide, giving rise to more mature cells bearing both IgM and IgD expressing the same idiotypic determinants which indicates that the two antibody molecules have the same or at least very similar sequences in the variable (V) region (153-154). Double producer cells (IgM-IgD) were found to constitute the majority of splenic B lymphocytes in several mammalian species including human (155), mouse (153-154), monkey (155), rabbit (156), and rat (157). With regard to density of surface Ig, B cells bearing both IgM and IgD have been shown to be heterogenous with respect to the density of the two isotypes on different subpopulations (152). In particular, the IgM/IgD ratio was found to decrease as B cells mature (158-159). At some point in this differentiation, some B cells bearing both IgM and IgD can undergo a "class switch" leading to expression of one of the other antibody classes (IgG, IgA or IgE).

Unlike other classes of immunoglobulins the level of IgD in the sera of different mammalian species is extremely low (155, 160-161).

The presence of IgD as a common B cell surface molecule coupled with its paucity in the serum has led to the suggestion that this isotype serves predominantly a receptor function. The presence of two

antigen-receptors on the same cell (namely IgM and IgD) which are acquired at different stages during ontogeny urged investigators to look for a unique function for surface IgD. IgD has been attributed a special role during early stimulation of the immune system by antigen (162-163) and in memory propagation (164). Since IgD is acquired roughly at the same time that neonatal B cells become less susceptible to tolerance induction it was suggested that IgD may play a central role in tolerance prevention (165-166) although contradictory observations have recently been reported (167-168). Recent studies have suggested that cross-linking of receptors is a prerequisite for inducing proliferation of B cells via Ig receptors (169-170) and that the epitope density of an antigen plays a critical role in determining the requirement for IgM vs. IgD on the responding B cells (171).

While none of the above hypotheses has been widely substantiated, it is at least accepted that IgD does have an active signaling role since it was demonstrated that anti-δ antibodies can trigger B cell proliferation both in human (172-173), rat (174) and most recently in the mouse system (175-176).

Primary structure of IgD; Has it been conserved during evolution?

Biochemical studies of IgD were restricted for a long time due mainly to difficulties in obtaining enough purified material. Not only is the level of serum IgD very low, but also until recently no IgD producing myelomas of rat or mouse origin were available. Similarly, the incidence of IgD myelomas in man is low, the survival time of the patients is short, and the serum concentration of the IgD

myeloma protein is usually low. Nevertheless, preliminary studies of human IgD revealed some interesting structural features (177-178). 1) δ-chain and especially its Fc region is very rich in carbohydrates. The human δ-heavy chain has a molecular weight suggesting either a five domain structure (179) or a four-domain structure with an "extended" hinge region (180). 2) The presence of a single inter-heavy-chain disulfide bond also appears to be unique among mammalian immunoglobulins (181). 3) Surface IgD molecules were found to be extremely sensitive to proteolysis (163).

Certain predictions concerning the conservation of IgD primary structure during evolution could be made, if we examine similar studies of the other ubiquitous B cell receptor - IgM. Phylogenetic, evolutionary and immunochemical data suggest that the IgM class of immunoglobulins has been more rigidly preserved in evolution than α, γ or light chains, since IgM molecules from different species cross react in serologic tests more extensively than do IgG, IgA, or light chains (159, 182). When the complete amino acid sequences of the Fc region of a canine and human μ chain were compared, an overall homology of more than 82% was found. When the different subregions were compared there appeared to be a gradient of increasing homology from the hinge region (68%) to the carboxy-terminus which is embedded in the cell membrane (92% homology) (183). It was postulated that preservation of IgM primary structure was dictated by the special function of this Ig class as a major B cell receptor. Thus the evolutionary selection pressure would act to preclude the kinds of amino acid interchanges which have occurred in the IgA and IgG classes.

It would be reasonable to predict that similar to IgM, the primary structure of the IgD receptor on B cells would be conserved in evolution. Especially those regions which are involved in membrane attachment of this molecule and those directly involved in the unique function of IgD as antigen receptor on B cells. In manuscript 1 of this thesis this prediction was tested using serologic and immunofluorescent techniques. The paper demonstrates the serologic cross reactivity between mouse and rat surface IgD and human serum IgD.

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PAPER 1.

Cross-reactivity of rat, mouse, and human IgD

Abstract

Highly purified sheep anti-rat lymphocyte membrane IgD (mIgD) was used to detect cross-reactivity with the putative murine-δ chain on mouse lymphocytes. Cross-reactivity is demonstrated by indirect immunofluorescent staining and by immunoprecipitation of ^{125}I-labeled lymphocyte membrane extracts followed by electrophoresis on 10% polyacrylamide gels. In addition, cross-reactivity of anti-rat with human IgD is shown by gel diffusion analysis.

The anti-rat-δ reagent stained both Ig5a$^+$ and Ig5b$^+$ lymphocytes. Preincubation of Ig5b$^+$ (but not Ig5a$^+$) cells with monoclonal allotype-specific antibodies (anti-Ig5b) under capping conditions caused inhibition of staining by the sheep anti-rat-δ reagent, indicating that it is the δ-chain that is recognized on mouse lymphocytes and that the anti-rat-δ reagent does not distinguish between mouse allotypes. Furthermore, absorption of the sheep anti-rat-serum with purified human IgD reduced subsequent staining of mouse lymphocytes by approximately 50%; staining was not affected by absorption with human IgM. This xenogeneic anti-δ antiserum appears to detect determinants on the δ-heavy chain, which are shared by at least three species of mammals, suggesting that these determinants represent important molecular features conserved during evolution.

Introduction

IgD is an important B lymphocyte surface immunoglobulin. However, because of a lack of available mouse IgD-secreting myelomas or readily

detectable serum IgD, characterization of the mouse counterpart of human IgD has depended on analysis of cell surface immunoglobulins by sensitive isotopic-labeling techniques that use sequential precipitation with heterologous anti-μ and anti-light chain antisera (1, 2). More recently, alloantisera against various allotypic variants of mouse IgD were produced by using spleen cells congenic at the Ig5 locus as immunogens (3). These antisera have been used in several biochemical and biologic studies of surface-bound IgD (4-6). The murine cell-surface candidate for IgD resembles the human counterpart in its marked susceptibility to proteolysis, and physicochemical properties (size, carbohydrate content) (7), but in the absence of amino acid sequence data, this new Ig class in the mouse remains to be identified formally as IgD.

In view of the unusual association of IgD with B cell membranes it might be expected that this class of Ig would show a high degree of conservation during evolution as does IgM. As a result, antigenic cross-reactions between IgD of various species might be readily detectable. In keeping with this view Ruddick and Leslie (8) reported antigenic cross-reactivity between human and rat IgD with a chicken anti-human IgD antiserum. In this paper we have used sheep anti-rat-δ antiserum to demonstrate an antigen on mouse lymphoid cells that cross-reacts with rat and human IgD. The isolated ^{125}I-labeled surface molecules from mouse lymphocytes have the electrophoretic properties of IgD, and serologic studies support the hypothesis that human, rat, and mouse IgD molecules have antigenic specificities in common.

Materials and Methods

Antisera. Sheep anti-rat membrane IgD was prepared as previously described (9). Briefly, rat lymphocytes (Sprague-Dawley, Madison, Wis) from peripheral blood and lymphoid organs were isolated on a Hypaque-Ficoll gradient. The cells were washed three times with PBS and lysed with 0.5% (v/v) NP 40, (Particle Data Laboratories Ltd., Elmhurst, Ill.) in PBS containing 1 mM phenylmethylsulfonyl fluoride at 25 C for 10 min. The lysate was centrifuged at 3000 X G for 30 min at 4 C, and the supernatant was passed over column immunoabsorbents (IA) containing normal sheep IgG and normal chicken IgY. The non-adherent material was then passed over an IA column conjugated with specifically purified chicken anti-human-δ (9). The adherent material was eluted with 3 M NaSCN, dialyzed, and concentrated; 890 µg emulsified in an equal volume of CFA were injected subcutaneously into multiple sites on a sheep. The sheep was boosted 4 weeks later with 450 µg in CFA. Several bleedings were collected starting 2 weeks after the boost. The IgG fraction of the antiserum so obtained was isolated by 40% saturated ammonium sulfate precipitation and a passage over a Sephadex G-200 column equilibrated in Tris HCl NaCl buffer (pH 7.4) The IgG fraction was concentrated by negative pressure and made δ-chain specific by sequential passages over immunoabsorbent columns conjugated with normal rat plasma, normal rat serum (pooled from several strains), normal chicken serum, rat IgG, and rat IgM. Before staining of mouse lymphoid cells, the antibody was also passed over separate IA columns conjugated with mouse IgG (MOPC 195, IgG2b, κ), IgM (MOPC 104 E, IgM λ_1). and mouse IgG (MOPC 315, IgA λ_2), and

finally, it was absorbed extensively with rat and mouse thymocytes. This reagent contained 23.2 mg protein/ml and 1.1 mg anti-δ Ab/ml, as determined by radial immunodiffusion with purified human IgD as a standard. Normal serum (NSS) from an unimmunized sheep was similarly treated and used as a control serum in all experiments.

Goat anti-human IgM (Lot No. 89929, 4.7 mg AB/ml) and goat anti-human IgD (Lot No. 88923, 4.7 mg Ab/ml) were obtained from Meloy Laboratories Inc. (Springfield, Va). Rhodamine-conjugated rabbit anti-sheep IgG (Lot No. 11482, R/p 3.5 mg/gm) was obtained from Cappel Laboratories, Downington, Pa.

Sheep anti-mouse-μ antiserum was produced in our laboratory by hyperimmunizing sheep with purified MOPC 104E (IgM λ_1) myeloma protein. The antiserum was made isotype specific by passages over IgG (MOPC 195, IgG2b κ) and IgA (MOPC 315, IgA λ_2) immunoabsorbent columns.

Purified human IgD and IgM immunoglobulins. Human IgD was isolated from the serum of an IgD myeloma-bearing patient by two successive 40% saturated ammonium sulfate (SAS) precipitations followed by passages over Sephadex G-200 and DEAE columns. The isolated protein contained 5 mg IgD/ml as determined by radial immunodiffusion and revealed only one precipitation band in immunoelectrophoresis (IEP) against anti-whole human serum antiserum.

Human IgM myeloma protein was isolated from the serum of an IgM myeloma-bearing patient by 40% SAS precipitation followed by precipitations in water (differential isolation of euglobulin fraction). The precipitated material was then chromatographed over Sepharose 4B

column equilibrated in Tris HCl NaCl buffer. The purifed IgM preparation contained 12.5 mg Ig/ml and gave a single band on IEP against an anti-whole human serum antiserum.

Monoclonal alloanti-mouse-δ. This reagent was obtained from Sera-Lab Ltd. (Crawley Down, Sussex Copthone, England). The specificity of this monoclonal antibody for the Ig5b allotypic determinant on mouse was previously described (10).

Mice. BALB/c mice were purchased from the Fred Hutchinson Cancer Center, Seattle, Wash. B10.A and B10.A(4R) strains were obtained from ARS Sprague Dawley.

Immunofluorescent staining of lymphoid cells. Cells to be stained were treated with 0.83% Tris-ammonium chloride for 5 min in a 37 C waterbath to lyse erythrocytes and were subsequently washed three times with PBS. For staining, they were resuspended in cold PBS + azide (0.03 M) and pelleted in 6 x 50 mm glass tubes, 3×10^6 cells per tube. Cells were incubated with 25 µl of the first antiserum (anti-δ) for 30 min on ice, washed twice with cold PBS-azide, and the rhodamine labeled developing serum was added for an additional 30 min at 4 C. Wet mounts were prepared after the cells received a final three washings in PBS-azide. At least 300 cells were examined by fluorescent microscopy with a Zeiss Universal microscope equipped with type III 12.5 epifluorescence condenser for two wave length narrow band excitation (Schares Instrument Corp., Houston, Tex.) When indicated, preincubation with alloanti-δ antiserum under capping conditions was included. In these experiments cells were suspended in

PBS without azide and after initial incubation with the antiserum at 4 C, were transferred to a 37 C water bath for an additional 45 min.

Cell surface iodination and co-precipitation of labeled surface Ig. Suspensions of rat or mouse spleen cells were labeled by the lactoperoxidase technique of Marchalonis (11) as previously described (8). Lysates from surface labeled mouse cells were first reacted with sheep anti-rat-δ, and sheep anti-mouse-μ or normal sheep serum. Lysates from labeled rat cells were similarly treated with sheep anti-rat-δ or goat anti-rat-μ specific antisera. After incubation for 1 hr at 37 C the complexes were precipitated by the addition of excess rabbit anti-goat IgG. (Rabbit anti-goat antiserum cross-reacts with sheep immunoglobulins in gel diffusion, unpublished observation.) The resulting precipitates were pelleted by centrifugation at 2500 X G for 20 min at 4 C and were washed five times with cold PBS. The supernatant from each wash was counted to assess removal of unbound reactivity.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed on the radioactive samples according to the method of Laemmli (12). The gels contained 10% polyacrylamide and 0.1% SDS. The samples were suspended in Laemmeli sample buffer (12) and were heated at 100 C for 2 min. After cooling, 1 μl of 0.05% (w/v) bromophenol blue was added as tracking dye. Samples containing a known amount of radioactivity were loaded onto the gels and electrophoresed in parallel with m.w. markers: Phosphorylase B (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), and myoglobin (17K). After electrophoresis, the gels were

frozen at -20 C and sliced. The ^{125}I-labeled fractions were counted in a Beckman Biogamma spectrometer (Beckman Instruments, Fullerton, Calif.).

Immunoabsorbents. Immunoabsorbent columns were prepared by covalently binding protein to Sepharose 4B (Pharmacia, Upsala, Sweden) by the CNBr method as described by Wofsy and Burr (13).

Immunodiffusion analysis. Ouchterlony tests were carried out in 1% agarose (Seakem, Maine) in sodium barbital buffer at pH 7.4.

Results

Specificity of sheep anti-rat membrane IgD (mIgD). The specificity of the sheep anti-rat-δ antiserum was examined by multiple criteria. The antiserum did not react with pooled normal rat serum, rat saliva, or purified rat IgG, IgM, or IgE as judged by immunodiffusion. To exclude the possibility that the serum contained some anti-μ reactivity too low to be detected by gel diffusion, the sheep anti-rat-mIgD was further tested in a radioimmunoprecipitation assay with ^{125}I-rat IgM. As can be seen in Table I the amount of radioactivity precipitated by this serum did not exceed background counts precipitated by normal sheep serum, which was approximately 1% of the counts precipitated by the anti-μ reagent.

In preliminary studies in the rat (not shown) that used this anti-δ reagent, the organ distribution and the number of δ^+ cells in various tissues closely correlated with those of μ^+ cells. Furthermore, μ and δ were shown to be co-expressed on a large percentage of

TABLE I

Lack of reactivity between sheep anti-rat IgD and purified rat IgM as determined by radioimmunoprecipitation assay[a]

Antiserum	Total Radioactivity of Precipitate (cpm)
Normal sheep serum	1,325 ± 453[b]
Sheep anti-rat IgD	1,142 ± 460
Goat anti-rat IgM	116,249 ± 7,076

[a]Purified rat IgM was ^{125}I-labeled by the lactoperoxidase method. Equal amounts of radioactive material were added to 10 µl of the various antisera. After incubation the immune complexes were precipitated by rabbit anti-goat antiserum at equivalence.

[b]Mean ± S.D. of four replicates.

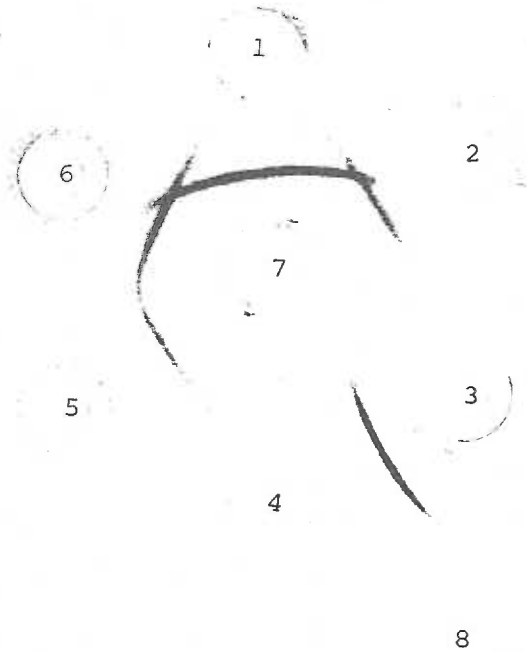
rat Ig^+ cells through their ability to be co-capped by anti-rat-Ig and to be capped independently by anti-δ and anti-μ antisera.

When rat spleen lymphocytes were stained for cytoplasmic immunoglobulin with fluorescein or rhodamine-conjugated antisera, only 0.3% of splenic lymphocytes stained with the anti-δ reagent as compared to staining of approximately 15% and 35% of the cells by anti-IgM and anti-rat Ig antisera, respectively. The reactivity of the sheep anti-rat-δ reagent with human and mouse isotypes was next examined (Fig. 1). A single line of precipitation was formed with human serum containing IgD that showed identity with the precipitate formed with goat anti-human IgD (Fig. 1a, b) but not with goat anti-human IgM. As can be seen in Figure 1b, no reactivity could be detected between anti-rat-mIgD serum and pooled normal mouse serum or with purified myeloma proteins; MOPC 104E (IgM, λ_1), MOPC 195 (IgG-2b, κ), and MOPC 315 (IgA, λ_2). These results suggest a specific cross-reaction between human IgD and the rat membrane IgD against which the antiserum was made.

Indirect immunofluorescent staining of mouse lymphocytes. Comparable percentages of spleen cells (20 to 30%) from several strains of mice were stained with sheep anti-rat-δ serum (Table II). The numbers of μ-bearing cells determined by staining with sheep anti-mouse-μ was usually higher than δ^+ cells (average 35%). Thymus cells showed a negligible degree of staining even before absorption of the antiserum with thymocytes.

Figure 1. Reactivity of sheep anti-rat membrane IgD by immunodiffusion analysis. A: well 1, goat anti-human IgM (μ specific); wells 2, 5, and 8, sheep anti-rat mIgD; well 3, normal rat serum; well 4, sheep anti-rat IgM (μ specific); well 6, goat anti-human IgD (δspecific); well 7, human serum containing 350 μg/ml IgD. B: well 1, goat anti-human IgD (δ specific); well 2, human IgD myeloma protein (1 mg/ml); well 3, normal mouse serum (pool); wells 4 and 6, purified MOPC 104E myeloma protein (IgM, λ_1 1.3 mg/ml); well 5, goat anti-mouse-IgM (Cappel Labs.); well 7, mixture (1:1) of MOPC 195 (IgG2b, κ 1.6 mg/ml) and MOPC 315 (IgA, λ_2 1.4 mg/ml); well 8, sheep anti-rat membrane IgD.

A



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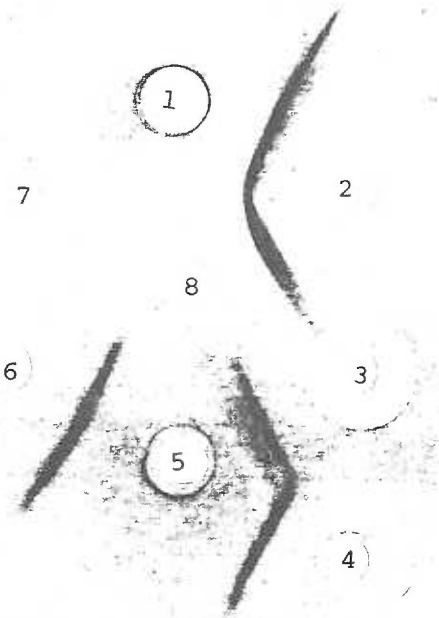


TABLE II

Percent of mouse lymphocytes staining with sheep anti-rat-δ antiserum[a]

Strain	IgD Allotype	% of δ^+ Cells	
		Spleen	Thymus
BALB/c	Ig5a	20 ± 6[b] (13-28)[c]	< 1
B10.A	Ig5b	26 ± 6 (20-33)	< 1
B10.A(4R)	Ig5b	25 ± 3 (23-28)	< 1

[a]Determined by indirect immunofluorescent staining. Rhodamine conjugated rabbit anti-sheep IgG was used as second antiserum in all experiments.

[b]Mean of two counts ($\geq$200 cells/count) ± S.E.

[c]Range of 2 to 3 experiments.

All the experiments included control samples that were reacted with normal sheep serum (NSS) followed by the rhodamine-conjugated anti-immunoglobulin reagent and others incubated only with the second antiserum. In both types of controls, no staining could be detected.

Capping with BALB/c anti-B10 allotype. If the sheep anti-rat-reagent indeed recognizes IgD molecules on the surface of mouse lymphocytes, one should be able to prevent staining by prior capping with specific alloanti-δ. This approach was taken by using monoclonal anti-Ig5b of BALB/c origin. The use of hydridoma monoclonal antibodies eliminates the problem of multiple specificities such as anti-H-2 and anti-δ reactivities, which have been found by others in conventionally raised alloanti-δ (14). The hybridoma anti-δ used here, H6/31.HLK (10) is specific for the Ig5b allotype and does not cross-react with mouse IgM (15). Pretreatment of B10.A (Ig5b) with the monoclonal anti-δ reagent under capping conditions before staining with sheep anti-rat-δ reduced the number of δ^+ splenic lymphocytes by 57 to 80%. Similar treatment of BALB/c (Ig5a) splenic lymphocytes had no effect on their ability to be stained with sheep anti-rat-δ (Table III); thymocyte staining (2%) was unaffected. These results strongly suggest that the sheep anti-rat IgD reacts with the δ-isotype on mouse lymphoid cells.

Cross-reactivity between human and rat was suggested from the gel diffusion analysis as shown in Figure 1. As a further means of determining whether cross-reactive determinants are shared by human, rat, and mouse-δ heavy chains, purified human IgD or IgM were added together with sheep anti-rat-δ during the first stage of

TABLE III

Allotype specific Antiserum (anti Ig5b) can selectively block staining of mouse Ig5b$^+$ spleen cells but not Ig5a$^+$ spleen cells with sheep anti-rat-δ antiserum

Strain	Expt. No.	Pre-Capping with Monoclonal Anti-Ig5b Antiserum[a]	% Cells Staining with Sheep Anti-rat-δ	%Reduction
BALB/c (Ig5a)	1	-	28 ± 4[b]	
		+	25 ± 3	11
	2	-	14 ± 0.2	
		+	19 ± 2	0
B10.A (Ig5b)	1	-	34 ± 3	
		+	7 ± 2	80
	2	-	20 ± 6	
		+	5 ± 2	75
Thymocytes[c]		-	2	
		+	2	0

[a]Cells were incubated with monoclonal anti-Ig5b antibodies for 30 min on ice followed by 45 min at 37 C to allow capping. Subsequent staining with sheep anti-rat-δ was conducted under noncapping conditions (4 C, 0.03 M NaN_3).

[b]Arithmetic mean of two counts (≥200 cells/count) ± S.E.

[c]B10.A thymocytes.

immunofluorescent staining. As shown in Table IV, purified human IgD blocks staining of mouse spleen cells with sheep anti-rat-δ by about 50%. Human IgM had no effect under the same conditions. Thus, it appears likely by this criterion as well, that at least some determinants are shared by human and mouse-δ heavy chains and can be detected by sheep anti-rat-δ antiserum. Cross-reaction between rat and human-δ chains was described previously with a chicken anti-δ reagent (8, 16).

SDS-PAGE analysis of immunoprecipitates. Figure 2 compares the membrane components precipitated by sheep anti-rat-δ from mouse and rat spleen lymphocytes, the radioactive profile obtained after reduction and electrophoresis of ^{125}I-labeled rat splenic lymphocyte lysates is similar to that obtained previously with chicken anti-δ (8). The δ-chain peak co-electrophoresed with rat-μ chain with the exception of a small shoulder at approximately 65,000 daltons (Fig. 2-I). In addition to the heavy and light chain peaks, one additional peak of 37 to 38,000 was precipitated consistently by this as well as by the previously described chicken anti-human IgD, rabbit anti-rat IgD, and nonimmune chicken and sheep serum (8, 9 and Fig. 2-I and III); this peak was not detected in lysates of mouse membranes. The identity of this material has not been determined. Precipitation of iodinated mouse spleen membrane proteins with the same sheep anti-rat-δ or sheep anti-mouse-μ antisera brought down heavy chain peaks distinguishable by their electrophoretic mobility; in contrast they brought down light chain peaks that migrated in the same relative position (Fig. 2-II). The calculated m.w. for the isotype peaks was

TABLE IV

Staining of mouse spleen lymphocytes by sheep anti-rat-δ antiserum is specifically blocked by human IgD (but not IgM) molecules

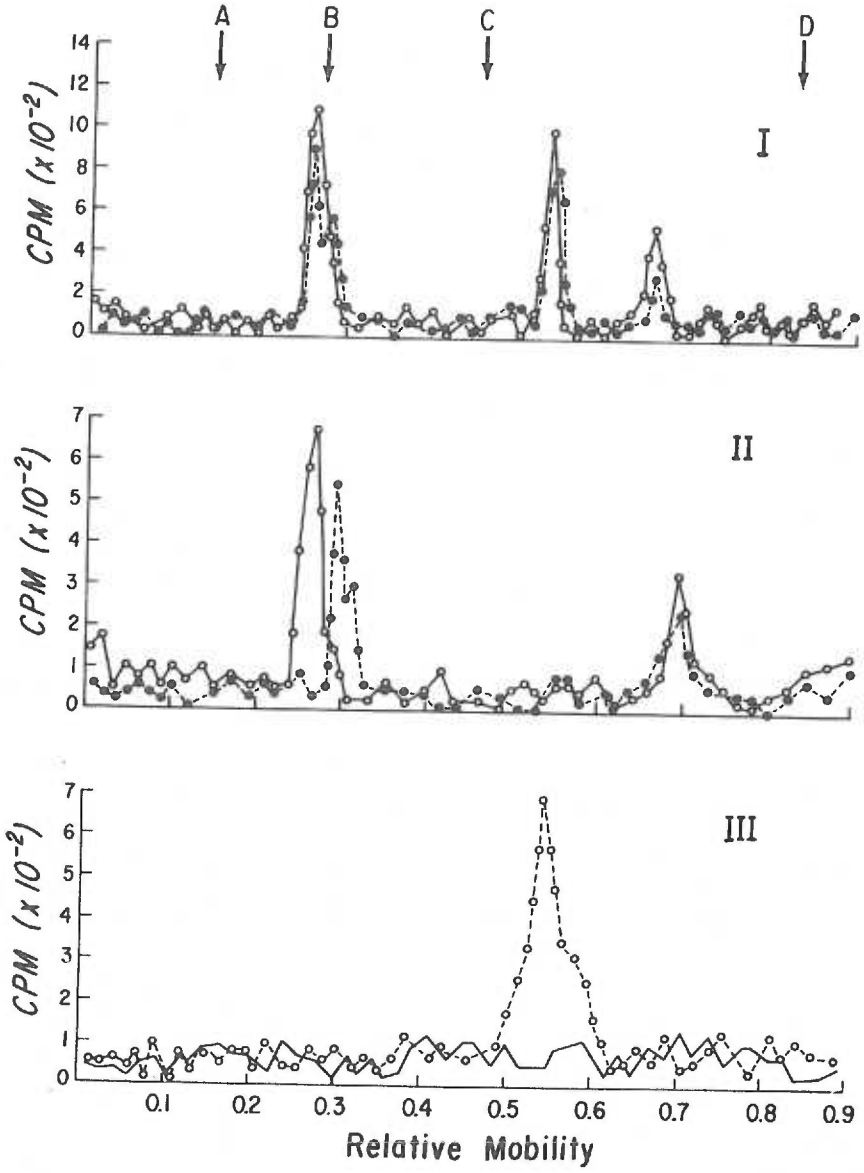
Strain	1st Antiserum: Sheep Anti-rat-δ in Presence of	% Lymphocytes Positively Stained	% Inhibition of Staining
BALB/c		12 ± 1.1[c]	
	Human IgD[a]	7 ± 3	42
	Human IgM[b]	12 ± 0.1	0
B10.A		19 ± 3	
	Human IgD	9 ± 3	55
	Human IgM	20 ± 2	0

[a]Mouse lymphocytes (3×10^6) were incubated in presence of 25 µl of purified human IgD (5 mg/ml) and 25 µl of anti-rat-δ serum for 30 min at 4 C.

[b]As above, but in presence of purified human IgM (12.5 mg/ml).

[c]Mean of two counts ($\geq$200 cells/count) ± S.E.

Figure 2. Precipitation of mouse and rat membrane IgD molecules by sheep anti-rat-δ antiserum SDS-PAGE analysis. I, precipitation of ^{125}I-labeled rat lymphocyte membranes extracted with: (o——o) goat anti-rat-μ serum; (●--●) sheep anti-rat-δ serum. II, precipitation of ^{125}I labeled mouse lymphocyte membranes extracted with: (o——o) sheep anti-104E (μ-specific); (●---●) sheep anti-rat-δ serum. III, precipitation of (o——o) mouse or (o---o) rat ^{125}I-labeled lymphocyte membranes extracted with normal sheep serum. Molecular weight markers: A, phosphorylase B (94K); B, BSA (67K); C, ovalbumin (43K); D, myoglobin (17K).


A
B
C
D
I
II
III
CPM (x10⁻²)
Relative Mobility
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

68 to 70,000 for the anti-μ reactive material and 62 to 65,000 for the anti-δ reactive heavy chain. These values are in good agreement with values obtained in other laboratories that used heterologous (17, 18) or alloanti-δ antisera (4, 6).

Discussion

The data presented in this paper demonstrate that membrane-bound IgD on mouse splenic lymphocytes can be recognized specifically by highly purified sheep anti-rat mIgD serum. Twenty to twenty-five percent of spleen lymphcytes (but not thymocytes) from various mouse strains could be stained with this reagent (Table II). Similar values were reported by others using antisera made against the putative mouse mIgD (18, 19). Furthermore, as shown in Table II monoclonal antibody specific for the mouse IgD allotypic determinant encoded by the Ig5b locus under capping conditions resulted in 80% reduction in the number of B10.A (Ig5b) but not BALB/c (Ig5a) lymphocytes subsequently stained by the rat-δ specific reagent. These findings strongly suggest that the sheep anti-rat-δ antiserum recognizes IgD molecules on the surface of mouse B cells that are cross-reactive with rat IgD molecules. The same reagent was also applied in immunoprecipitation of radioiodinated mouse lymphocyte membrane extracts. Electrophoresis of the precipitated material on 10% SDS-polyacrylamide gels under reducing conditions revealed only two peaks; one of 24,500 daltons, which corresponds to mouse light chain, and a second, slower migrating peak, which migrated slightly faster than the μ-heavy chain peak and corresponded to a 63 to 65,000 dalton component(Fig. 2b). The profile

obtained closely resembles those described by other investigators who have isolated the putative mouse IgD using heterologous or homologous antisera (reviewed in 4 and 20).

The anti-rat IgD does not distinguish allotypic variants of mouse IgD since three different strains of mice representing the two IgD allotypes described by Goding and colleagues (3) showed a very similar percentage of IgD^+ cells (Table I). It seemed likely, therefore, that the cross-reactive determinant(s) on rat and mouse IgD were highly conserved and would be shared by more than these two species. We tested this hypothesis by trying to block staining of mouse lymphocytes by the anti-rat-δ reagent with purified human IgD or IgM molecules. As expected, a reduction in the number of staining cells was achieved only in the presence of human IgD (5 mg/ml) but not IgM (12.5 mg/ml) molecules. The partial inhibition achieved (approximately 50%) suggests that only partial cross-reactivity exists between human and mouse IgD; however, in these experiments no attempt was made to absorb exhaustively with human IgD. The sheep anti-rat-δ serum probably contains several populations of antibodies recognizing different determinants on the IgD heavy chains. Some of these determinants are obviously shared between rat and mouse (Tables II and III, Fig. 2) and between rat and human (Fig. 1), but only a fraction of these would be expected to be shared by all three species. Alternatively, the failure to achieve complete blocking by human myeloma IgD may suggest that the sheep anti-rat-δ reagent recognizes more than one subclass of IgD.

Recently, Bazin et al. (21) described a heterologous antiserum raised against a rat myeloma protein that differs from most rat heavy

chain isotypes. The antiserum stained rat B cells and precipitated two membrane components of somewhat lower m.w. than those reported by Ruddick and Leslie (8). This putative anti-rat-IgD also stained mouse lymphoid cells, but the nature of the mouse surface determinants was not examined.

Phylogenetic and immunochemical data, including primary structure determinants, suggest that IgM has been more rigidly conserved in evolution than κ, λ, γ, or α chains (22-24). Greater than 82% homology was demonstrated between canine and human IgM by Wasserman and Capra (24). These findings are particularly intriguing in view of the fact that this isotype is generally viewed as being the first one to appear during evolution. Being a major receptor on B cells probably dictated structural requirements of this class of immunoglobulins and highly restricted the amino acid interchanges allowed in the Fc portion of these molecules. Similarly, IgD molecules represent a major B cell receptor in all the species in which this Ig class has been identified. Thus far, an IgD-like molecule has been reported in the mouse (1, 2), rat (8, 21), monkey (15, 25, 26), chicken (16), rabbit (27), and tortoise (28). Thus, it is reasonable to use arguments similar to those above for the evolutionary conservation of IgD. We would predict, therefore, that once the primary structure of IgD immunoglobulins from different species are available, a high degree of homology will be readily demonstrated.

Recently IgD-producing placmacytomas of murine (29) and rat (30) origin were identified and large scale isolation and biochemical analysis of their products was conducted. In addition two δ-chain

cDNA probes, one corresponding to the genomic DNA in BALB/c mouse liver (29) and a second corresponding to its "processed" transcriptional product from the murine IgD myeloma TEPC 1017 (31), were used in nucleotide sequence analysis (31). The DNA and amino acid sequences indicate an unusual structure for the murine δ-chain in two respects: 1) only two constant (C) region domains, termed (Cδ1) and (Cδ3) from homology considerations, were found (32). The two domains are separated by an unusually long hinge region (CδH) that lacks cysteine residues and thus cannot provide the covalent cross links between heavy chains typically seen in immunoglobulins. The two domains and hinge are all coded on separate exons. 2) at the carboxyl end of the δ chain, there is a stretch of 26 amino acids that is coded by an exon located 2750 to 4600 base pairs downstream from the rest of the gene. Analogy with IgM genomic DNA arrangement (33) suggests that this distally coded segment (CδDC) may have a membrane binding function; however, it is only moderately hydrophobic. A fifth potential exon, located adjacent to the 3' carboxy end of the Cδ3 could code for a stretch of 49 amino acids which is very polar and may be analogous to a similar stretch found in the carboxy terminal of secreted but not membrane bound μ chains.

The sequenced portion of the δ protein revealed seven potential carbohydrate binding sites (Asn-x-ser or Asn-x-ser) which explain why measurements of the size of IgD chains in gels have been markedly influenced by carbohydrate moieties which decrease electrophoretic mobility and led to higher m.w. estimates. From the amino acid compositions of Cδ1,, CδH, Cδ3 and CδDC a molecular size of 30,040 daltons

was calculated for the constant region of the TEPC 1017-δ chain. Addition of 14,000 daltons for typical V_H region and 1,000 daltons for an amino terminal leader segment of 10-15 amino acids brings the molecular size up to 44,000 daltons which is in agreement with the size of δ chain synthesized in vitro in a cell free system, or with the 43,000 dalton δ chain made in vivo in the presence of tunicamycin (34). Previous estimates of the molecular size of membrane IgD ranged from 62,000 to 72,000 daltons for both normal B cells and TEPC-1017 membrane IgD. Thus heavy post-translational glycosylation of the chain must be invoked to account for a final product of approximately 20,000 daltons greater molecular size.

The most unique features of the murine δ chain, namely, the absence of a whole domain (Cδ2) and the extended hinge region were also recently found to be the properties of rat IgD myeloma protein IR-731 (35). Biochemical analysis including amino acid sequencing of the purified δ-chain revealed a polypeptide of approximately 38,000 dalton (+13,000 dalton worth of carbohydrate) with a deletion of one domain in the Fc portion. Based on homologies with other immunoglobulins constant region domains it was concluded that most of the second domain (Cδ2) was missing. The amino acid sequence also revealed, in agreement with the mouse IgD data, an extended hinge region which exceeds 32 residues and contains a cluster of basic amino acids close to its carboxy terminus. This density of charged amino acids would easily account for the extreme susceptibility of the region to trypsin-like enzymes. Thus, the mouse and the rat IgD heavy chains share the same unusual primary structure which differs not only

from other immunoglobulin heavy chain classes but also from their human IgD counterpart, which contains three constant region domains (36).

In total, the cumulative data concerning the primary structure of human, rat and mouse δ-heavy chains reveal a pattern similar to that observed in our serological and immunofluorescent studies; sheep anti-rat IgD serum stained 20-25% of mouse splenic B-lymphocytes. This staining could be blocked (80% reduction) by precapping with the relevant allotype specific antiserum, but only partially blocked (50%) by purified human-IgD myeloma protein. While the degree of cross reactivity and interspecies homology would depend mainly on primary amino acid sequences (and such comparative studies have not yet been conducted), it is also likely that the absence of a whole domain in the constant region of both mouse and rat δ-chains could cause similar alterations in tertiary structure and therefore in antigenic determinants of these two species that would cause them to differ from that of the three-domain-containing human δ chain.

The main question is whether the Cδ2 is actually present in the genomic DNA but remains dormant in the tumors studied because of some "domain skipping mutation" which affects either the transcription or the processing of δ-mRNA. This hypothesis is difficult to accept since an identical deletion was found in two murine IgD tumors TEPC 1017 and TEPC 1033 and a similar one in the rat IgD myeloma (as discussed above). Moreover, the evidence that normal B cells have membrane δ chains similar in size to that of TEPC 1017 (34) suggests that these tumors express a normal form of the δ gene and not

an abberant one. The other alternative is that the gene for the second domain of δ-constant region is actually missing. The available nucleotide sequence data of the genomic introns seem to support this possibility since no obvious domain-like sequence, homologous to the human Cδ2 domain was revealed. If in fact both the mouse and the rat have lost the gene coding for the second domain of the δ-chain while the human retained it, this event must have taken place within the last 70 million years since rodents and humans diverged. It would be of interest to determine whether all rodent-IgD chains lack the Cδ2 domain or only some of them and relate it to the evolutionary trees constructed thus far.

Possible Structure-function Relationships. One of the unique features of the IgD heavy chain which is shared by human, mouse and rat is the unusual hinge region. In contrast to μ-chains which lack hinge region (32), the hinge region in the δ-chains of all 3 species is: 1) long (30-50 a.a); 2) rich in charged amino acid which form tryptic-cleavage sites; 3) low in proline residues and; 4) does not form inter chain disulfide bridges due to lack of the necessary cysteine residues. These properties of the δ-hinge region in addition to rendering the membrane IgD receptors extremely sensitive to proteolysis, may also allow IgD to be cross linked on the membrane by polyvalent antigens more efficiently than membrane IgM which lacks hinge region and contains two inter-μ chain disulfide bridges as mentioned above. There is suggestive evidence that membrane IgD is more effective than IgM in triggering B cells by thymus-dependent antigens that have a low density of antigenic determinants (37). Using various preparations of

trinitrophenylated-polyacrylamide beads (TNP-PAB) differing in their epitope density, it was found that while all TNP-PAB responding B cells express IgM and IgD on their surface, only the response to the low epitope density antigen was both T-dependent and required triggering signals from both surface isotypes. On the other hand, the response to the high epitope density antigen, was found to be both T-independent and IgD-independent. The ability of high epitope density antigens to trigger B cells in the absence of IgD suggests that such antigens can effectively cross-link the IgM receptors. In contrast, antigens of lower epitope density may be incapable of accomplishing the required cross-linking (38) without interacting with surface IgD. This explanation implies a major difference between the two isotypes in their ability to be cross-linked by antigens. The differences could reside in the density, flexibility, valency, or mobility of membrane IgM compared with membrane IgD.

Another phenomenon which is probably related to the lack of inter disulfide bonds in the hinge region is the presence of so called half IgD molecules (HL) on the surface of some splenic B cells in addition to the common H_2L_2 form (39, 40). These two noninterconvertible forms of surface IgD could be the products of two alternative carboxyl terminal exons, one with a Cys residue in the CδCD region and the other without. The same effect could also be accomplished by more elaborate Cδ domain switches. The hybridization studies published so far did not indicate the presence of 2 copies of δ per chromosome but it is possible that the technique is not sensitive enough to distinguish between one and two copies. Whatever

the mechanism responsible for the presence of the two molecular form on B cells the main question is whether they can both bind antigens and whether they deliver identical or opposite types of signals to the interiors of the developing B cells.

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PAPER 2.

Differential sensitivity of IgG memory subpopulations to allogeneic thymocytes: positive and negative signals

Abstract

Spleen cells from mice primed to trinitrophenyl keyhole limpet hemocyanin (TNP-KLH) can generate anti-TNP IgG plaque-forming cells when stimulated *in vitro* with either T-dependent (TD) or T-independent (TI) conjugates of the TNP epitope. The cells responding to TD or TI antigens can be eliminated selectively by exposure to antigen, bromodeoxyuridine, and light. These functionally distinct subpopulations of memory B cells have been tentatively designated B2γ (TD responders) and B1γ (TI responders). We have found using an *in vitro* "allogeneic effect" model that secondary responses to TD and TI antigens were modified differentially by alloreactive thymus cells. The TD response was highly sensitive to "negative signals" (50 to 90% suppression), whereas TI IgG responses were resistant to suppression and were frequently enhanced by the presence of allogeneic T cells. Limiting dilution analysis in the presence of allogeneic thymocytes showed a reduction in the frequency of B2γ precursors and an increase in the frequency of B1γ precursors under the same experimental conditions. Pretreatment of the added allogeneic thymocytes with mitomycin C eliminated the suppression of the TD response but did not alter the response to the TI complexes.

To define the immediate target of the alloreactive thymocytes, successful cooperation was established between mitomycin C treated KLH-primed T cells and TNP-primed B cells from partially histoincompatible congenic strains. Unprimed thymocytes were then added, which could only recognize as foreign either the B cell or the T-helper cell. Only thymocytes allogeneic to the primed B cells pro-

duced the differential effects described above. No effect was observed when the T helper cells were the allogeneic target.

Introduction

Priming mice with the thymus-dependent (TD) antigen trinitrophenyl keyhole limpet hemocyanin (TNP-KLH) elicits hapten-specific IgG memory cells, which produce a secondary IgG plaque-forming cell (PFC) response after challenge *in vitro* with either TD or thymus-independent (TI) forms of TNP (1, 2). Recent work has demonstrated that the IgG memory precursors that respond to these different forms of the hapten comprise at least two functionally distinct subpopulations, B1γ and B2γ, which can be selectively eliminated by bromodeoxyuridine and light treatment after stimulation by TI or TD antigen, respectively (3, 4). B1γ and B2γ give additive antibody responses and additive precursor frequencies after simultaneous challenge with certain TI and TD antigens (3, 4). Although these B1γ and B2γ subpopulations have been identified, their origins, relationship to one another, and the regulatory pathways that control them are undefined.

Positive and negative regulatory effects on B cells have been ascribed to different subsets of T lymphocytes in the mouse. Some regulatory T cells are antigen specific (5, 6) whereas others appear to function through recognition of nonantigen-related surface determinants. Thus, B cell expression may be modulated by regulatory cells that recognize idiotype, allotype, or isotype specificities (7-10), while other regulatory cells recognize major histocompatibility complex (MHC) encoded components (11, 12). Alloreactive T cells

have been shown to exert either positive (13, 14) or negative (14, 15) effects on antibody responses; however, these studies were seldom conducted so as to distinguish whether B cell subsets were differentially affected. Thus, we were interested in determining whether B1γ and B2γ cells responding to TNP differ in their susceptibility to such immunoregulatory mechanisms. In this paper we describe the differential sensitivity of B1γ and B2γ memory cells to allogeneic effects exerted in culture by unprimed allogeneic thymocytes. The IgG response to the TD antigen TNP-KLH was consistently suppressed whereas responses to the TI complexes TNP-T_4 and TNP-Ficoll were not suppressed and were often enhanced by the presence of the allogeneic cells; the target of the alloregulatory cell was in each instance a B cell.

Materials and Methods

Mice. BALB/c, B10.A(5R) and B10.129(5M) [the latter carries the H-2^b haplotype of the B10 strain but differs from B10 at the H-1/albino locus on chromosome 4 (16)] were obtained from Charles River Breeding Labs, Wilmington, Mass. Strain B10 was obtained from Simonson Laboratories, Gilroy, Calif. and B10.A from ARS Sprague-Dawley, Madison, Wis. Mice of both sexes were used but not in the same experiment; they were from 2 to 10 months of age when used.

Antigens. Trinitrophenyl-T_4 bacteriophage (TNP-T_4) was prepared as described previously (17). KLH was trinitrophenylated according to the method of Rittenberg and Amkraut (18) and had a mole ratio of TNP_{1057}-KLH based on a m.w. of 8×10^6 for KLH.

TNP was conjugated to Ficoll via its dichlorotriazine derivative according to the method described by Blakeslee and Baines (19) and had a molar ratio of $TNP_{9.1}$-Ficoll if it is assumed that Ficoll has a m.w. of 400,000.

Immunization. TNP-KLH was adsorbed onto bentonite (Fisher Scientific) according to the method of Gallily and Garvey (20) as modified previously (21). Mice were injected i.p. with 100 μg of TNP-KLH-bentonite in 0.5 ml saline on 3 successive weeks and were rested for at least 6 weeks before secondary challenge *in vitro*.

Cell cultures. Spleen cells were cultured in micro Mishell-Dutton cultures (22) as described previously (2). Each microculture contained 1 to 1.3×10^6 cells/well and the contents of four to eight such wells were pooled for assay as one culture. Three such cultures were assayed per experimental point to obtain data for statistical analysis.

Detection of anti-TNP response. All cultures were harvested on the 7th day of culture and assayed (23) for TNP-plaque forming cells (TNP-PFC) by using TNP-sheep erythrocytes (TNP-SRBC) (21). Cells producing IgG anti-TNP antibodies were detected by adding goat anti-mouse IgG antiserum and guinea pig complement (C) in the presence of a suppressive amount of anti-IgM (24).

Limiting dilution analysis. Limiting dilution experiments were performed with slight modifications of the system described by Quintans and Lefkovits (25). Hapten-primed B cells were depleted of T cells by treatment with rabbit anti-mouse brain antiserum (Accurate

Chemical and Scientific Corporation, Cedarlane Laboratories, Hicksville, N.Y.) and rabbit "low tox" C (Cedarlane); this is referred to as anti-θ + C treatment in the text. Graded numbers of T cell-depleted primed B cells were cultured in individual wells of Falcon No. 3034 tissue culture trays together with 5×10^4 KLH primed and mitomycin C-(Sigma Chemical Co., St. Louis, Mo.) treated spleen cells as a source of helper T cells (15). The total number of cells per well was kept constant (2×10^5 cells per well) by adding unprimed syngeneic thymocytes as filler cells. Cultures were stimulated with various antigens as indicated and the numbers of responding wells were determined on day 7 of culture by hemolytic spot test of culture supernatants on TNP-SRBC in 0.7% agarose, as described previously (4). Detection of IgG antibody was facilitated by flooding the plates with goat antimouse IgG antiserum. At least 60 wells were assayed for each experimental point. The frequencies of B1γ and B2γ memory precursors were calculated by Poisson statistic as previously described (4).

Source of allogeneic cells. Allogeneic T cells were obtained from thymuses of 6- to 10-week old, unprimed animals of various strains, as indicated in the text.

Results

The negative effects exerted by allogeneic T cells on secondary antibody responses have been previously analyzed (12, 15, 26, 27). Most of these studies, however, have been limited to TD responses or to IgM TI responses; thus, it was of interest to examine the effects

of allogeneic thymus cells on *in vitro* secondary IgG responses to TD and TI antigens.

As seen in Table I, when 1 x 10^5 allogeneic B10.129 thymocytes ($H\text{-}2^b$) were added at the initiation of secondary cultures of TNP-primed BALB/c spleen cell ($H\text{-}2^d$), the IgG response to TNP-KLH was suppressed by approximately 50%. On the other hand, generation of IgG PFC in response to the antigens TNP-T_4 and TNP-Ficoll was enhanced under the same conditions. Similar results were obtained on day 6 through day 9 of culture (not shown), indicating that these results were not due to a shift in the kinetics of either response.

Treatment of allogeneic thymocytes with mitomycin C before their addition to the cultures abolished the suppressive effect. This is in keeping with the observation of Swain et al. (15) that intrasplenic allogeneic suppressors of secondary TD responses are sensitive to mitomycin C. This treatment, however, did not alter the elevated responses to the TI comlexes, suggesting that the positive effect may be mediated by a separate population of alloreactive thymocytes not sensitive to treatment with mitomycin C.

Limiting dilution analysis. The two opposite allogeneic effects observed in the TD and TI-stimulated cultures could have resulted from a decrease in the burst size of B2γ memory cells and an increase in the burst size of the B1γ population or from changes in the actual numbers of B1γ and B2γ precursors triggered. Consequently, we carried out frequency analyses of the memory precursors to determine whether

TABLE I

The differential allogeneic effect of B10.129(H-2^b) thymocytes on secondary in vitro responses of BALB/c(H-2^d) B1γ and B2γ cells

Thymocytes Added[a]	Antigen Added in Culture		
	TNP-KLH	TNP-T_4	TNP-Ficoll
	IgG PFC/10^6	IgG PFC/10^6	IgG PFC/10^6
BALB/c (ddddddddd)	3413 ± 375[b]	2647 ± 362	1080 ± 146
B10.129 (bbbbbbbbb)	1535 ± 183	3623 ± 199	2319 ± 272
B10.129 mitomycin C-treated[c]	3305 ± 524	3254 ± 143	2770 ± 126

[a] 1 x 10^5 unprimed thymocytes were added to 1 x 10^6 TNP-KLH primed BALB/c spleen cells. Antigen was added and the cells were cultured for 7 days as described in Materials and Methods. Antigen doses were optimal: TNP-KLH 0.002 μg/ml; TNP-T_4 2.4 x 10^4 PFU/ml; TNP-Ficoll 0.01 μg/ml.

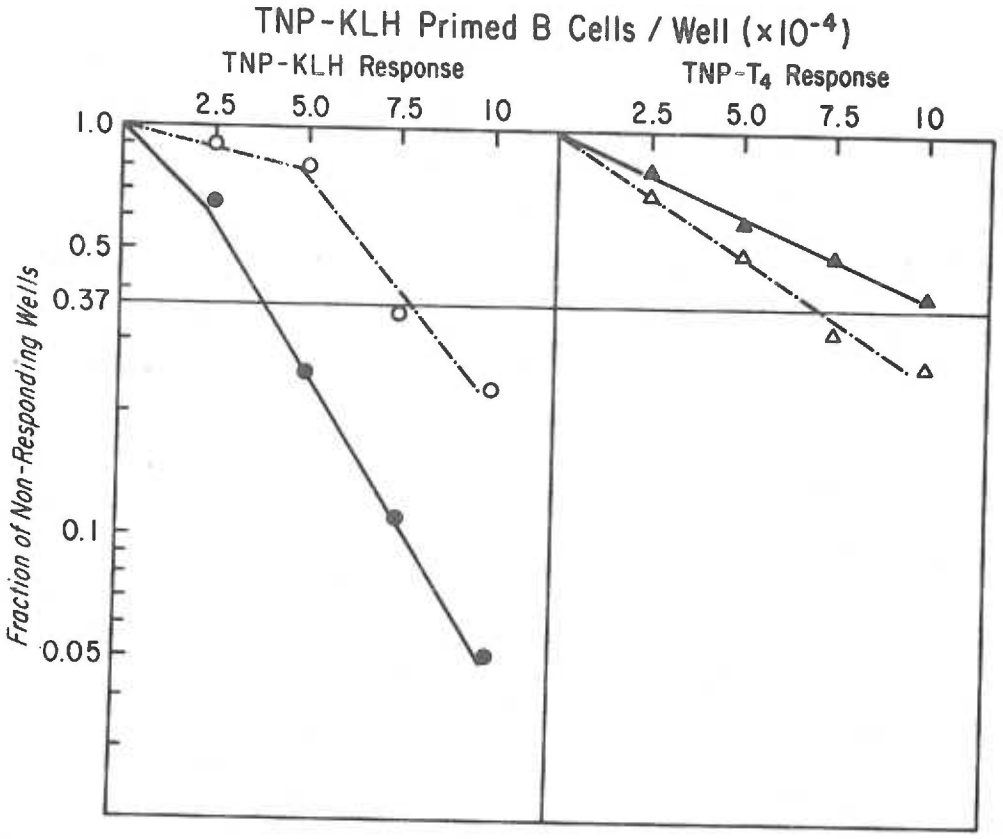
[b] Mean ± standard error of triplicate cultures assayed on day 7.

[c] Thymocytes were treated with mitomycin C (25 μg/ml) for 30 min at 37 C, washed three times and cultured with TNP-primed cells at the same cell ratios as above.

they were directly affected by the alloreactive cells. The results of one of three such experiments that all showed similar results are depicted in Figure 1. The solid lines demonstrate titration curves obtained when graded numbers of primed B cells were titered against an optimal number of KLH primed T-helper cells and stimulated with TNP-KLH (left panel) or TNP-T_4 (right panel). Cell density was kept constant (2×10^5 cells/well) by adding unprimed syngeneic thymocyte filler cells. The broken lines demonstrate titration curves obtained in the presence of 5% allogeneic thymocytes. As can be seen, the latter shifted the B cell titration curves for both TNP-KLH and TNP-T_4 responses but in opposite directions. The numbers of responding wells decreased in the TNP-KLH and increased in the TNP-T_4 cultures at all B cell concentrations. The calculated frequencies in the TNP-KLH stimulated cultures were $2.5/10^5$ B cells in the syngeneic mixture and $1.3/10^5$ B cells in the allogeneic mixture (50% decrease); for TNP-T_4 the frequencies were $0.9/10^5$ B cells in the syngeneic mixture and $1.5/10^5$B cells in the allogeneic mixture (66% increase). The reason for the biphasic curve (which suggests cell interaction) for TNP-KLH when allogeneic thymocytes were added is not clear but may indicate an insufficiency of allosuppressors at the higher B cell numbers. If allogeneic suppressors are monogamous as suggested for helpers by Waldmann et al. (28), then the presence of excess precursor B cells in a well could overcome allosuppression resulting in a positive well; Corley et al. (14) recently made the same suggestion.

As reasoned above, it appeared likely that 5% allogeneic thymocytes was less than optimal for maximal effects. Consequently, we

Figure 1. Plots of fractions of nonresponding wells obtained when B cells from TNP-KLH-primed BALB/c spleens were challenged *in vitro* with TNP-KLH (left panel) or TNP-T_4 (right panel) in absence or presence of allogeneic thymocytes. The anti-θ + C treated primed B cells were titered into wells containing 5 x 10^4 KLH-primed and mitomycin C-treated syngeneic helper cells. Total cell number (2 x 10^5/well) was kept constant by adding unprimed thymocytes either syngeneic (solid lines) or a mixture of syngeneic and 1 x 10^4 allogeneic thymocytes from B10.129 mice (broken lines). Sixty wells were assayed per experimental point.


TNP-KLH Primed B Cells / Well (x10⁻⁴)
TNP-KLH Response
TNP-T4 Response
2.5
5.0
7.5
10
2.5
5.0
7.5
10
1.0
0.5
0.37
0.1
0.05
Fraction of Non-Responding Wells

titrated increasing numbers of allogeneic thymocytes into cultures containing a constant number of primed B cells (5×10^4 per well) using mixtures of syngeneic and allogeneic thymocytes in different ratios and keeping the total number of thymocytes constant. The results in Table II show that in the presence of increasing numbers of allogeneic thymocytes the frequency of B2γ precursors responding to TNP-KLH decreased gradually to 28% of the value obtained in the absence of allogeneic cells. In contrast, under the same conditions, the frequency of TNP-T_4 responsive B1γ precursors increased 3-fold. Similar results were obtained in two other experiments. These data strongly suggest that the allogeneic effects observed resulted from changes in the total numbers of TNP specific B1γ and B2γ precursors expressed; however, they do not exclude that in addition, the burst sizes of the responding populations were affected.

Identification of the target of allogeneic thymocytes. It has been reported previously that allogeneic effectors act on B cells (27); however, it was necessary to ask this question here since a differential suppression of TD responses could also be obtained if the T-helper rather than the memory B cell was the immediate target of the suppression. The strategy adopted, as illustrated in Figure 2, was to establish cooperation across a partial histocompatibility barrier by using various combinations of congenic mice as donors of hapten-primed B cells or carrier-primed T cells that could then serve as distinct targets for allogeneic effector cells. Helper cells were obtained from spleens of KLH-primed mice and were treated with mitomycin C. Such cells contain T_H but lack allosuppressor cells and functional

TABLE II

Differential effect of nonimmune allogeneic thymocytes (B10.129, H-2^b) on BALB/c(H-2^d) B1γ and B2γ precursor frequencies

Thymocyte Combinations/Well[a]			
Syn: 10 x 10^4	Syn: 9.5 x 10^4	Syn: 9 x 10^4	Syn: 8 x 10^4
Allo: -	Allo: 0.5 x 10^4	Allo: 1 x 10^4	Allo: 2 x 10^4

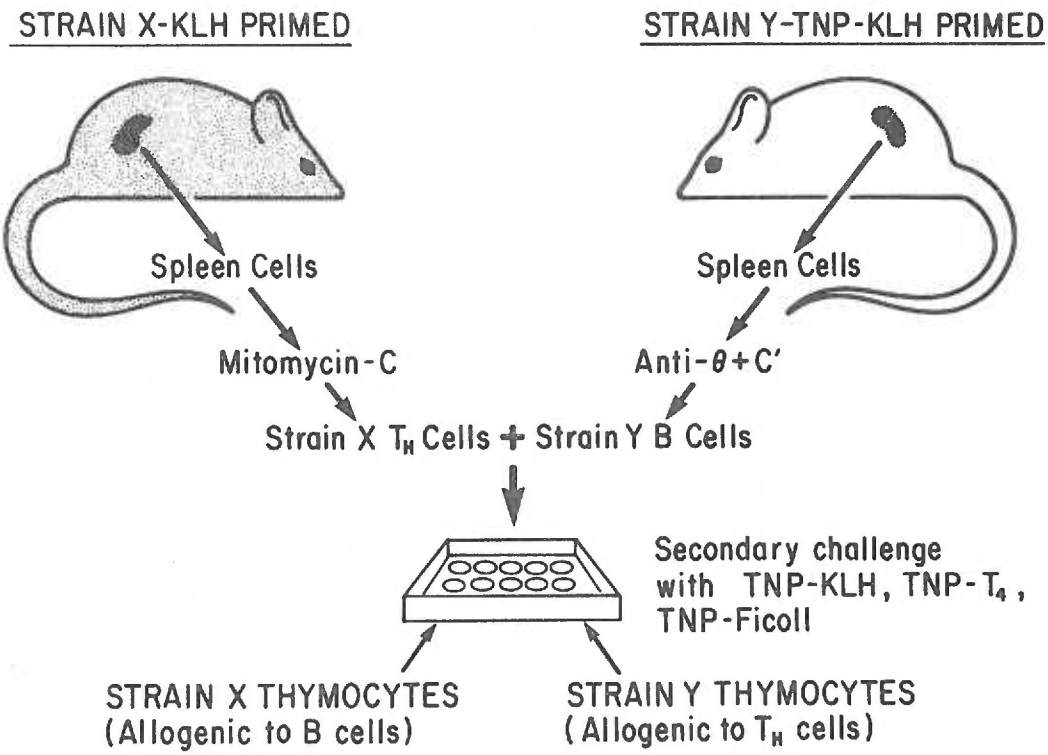
Antigen	No. of TNP-specific IgG precursors/10^5 primed B cells			
TNP-KLH	1.8[b]	1.0	0.6	0.5
TNP-T_4	0.7	1.7	2.5	2.2

[a]Each well contained 5 x 10^4 anti-θ + C-treated TNP-KLH-primed B cells, 5 x 10^4 mitomycin C-treated, KLH-primed, syngeneic spleen cells and 10 x 10^4 normal thymocytes. Antigen was added and the cells were cultured for 7 days. Antigen dose was optimal: TNP-KLH 0.002 μg/ml; TNP-T_4 2.4 x 10^4 PFU/ml.

[b]Sixty wells were assayed per experimental point. The frequencies of TNP specific precursors were calculated from the fraction of nonresponding wells according to Poisson distribution as described in Materials and Methods.

Figure 2. Experimental strategy to establish cell cooperation across partial histocompatibility barriers in order to identify the target of allogeneic effector cells. X and Y represent various congenic strains described in the text.

Establishment of Cooperation Across Histocompatability Barrier



PFC (15). Cells treated in this way whether hapten or carrier primed usually contain about 15% Ig^+ cells but do not respond to primary or secondary challenge with TD or TI antigens (unpublished observation).

Table III demonstrates successful cooperation between B10.A (kkkkkdddd) helper cells and B10.A(5R) (bbbkkdddd) B cells, which differ at the K, I-A and I-B regions of the H-2 complex. Multiple T_H:B ratios were tested, and the results at the optimal ratio are presented. We were able to establish successful cooperation using various strain combinations by the same experimental design. The reconstituted TD responses usually reached 50 to 80% of those obtained by the syngeneic T_H:B cell combinations. Reconstitution with unprimed congenic T cells was unsuccessful ($\leq$10% of control responses; data not shown) indicating that the help observed was of a carrier-specific type and not the result of a positive allogeneic effect on the B cells. Table III also illustrates the TD nature of TNP-KLH and the TI nature of TNP-T_4 in this system.

Having established that cooperation could occur across histocompatibility barriers under optimal *in vitro* conditions we could now add thymocytes allogeneic to either the B cells or the helper cells in order to identify which cell type was the immediate target of the alloregulatory cells. B10.A(5R) B cells were mixed with B10.A T_H cells and various numbers of unprimed thymocytes from B10.A (Fig. 3 top) or B10.A(5R) (Fig. 3 bottom) were added at the initiation of culture to the cell mixture. B10.A thymocytes being syngeneic to the helper cells could only recognize foreign MHC determinants carried by

TABLE III

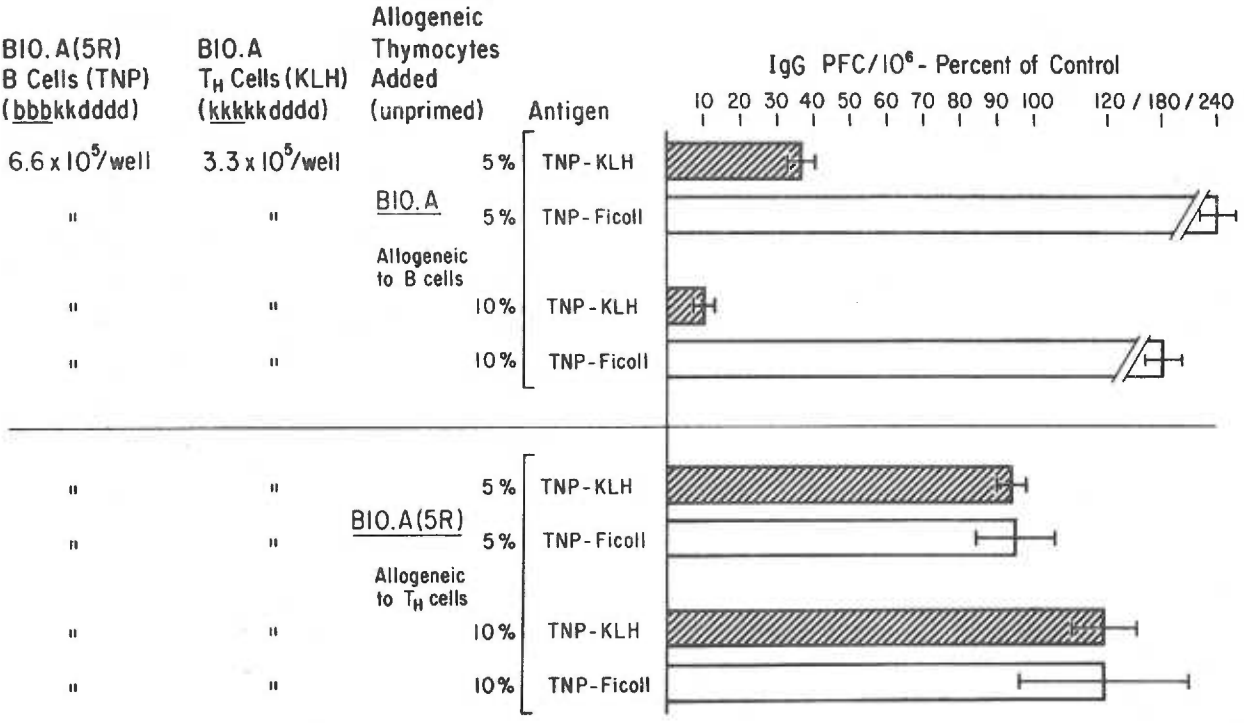
Cell cooperation across partial histocompatibility barrier in secondary *in vitro* T-dependent response

Responding Spleen Cells[a] B10.A(5R) (bbbkkdddd)	Anti-θ + C Treatment	T-Helper Cells[b] B10.A (kkkkkdddd)	MHC Diff.	TNP-KLH IgG PFC/10^6	TNP-T_4 IgG PFC/10^6
$10x10^5$/well	-	-		892 ± 124	2143 ± 514
$10x10^5$/well	+	-		26 ± 13	2100 ± 251
$6.6x10^5$/well	+	$3.3x10^5$	K,I-A,I-B	702 ± 88	1691 ± 257

[a]Spleen cells from mice primed with TNP-KLH. Culture conditions and antigen doses as in Table I.

[b]Helper cells from mice primed with KLH. The cells were treated with mitomycin C as described in Table I.

Figure 3. B cells are the target of alloreactive cells *in vitro*. B10.A(5R) TNP-primed B cells and B10.A KLH-primed helper cells were treated and mixed as described in Figure 2 and Table III. 5% or 10% normal thymocytes of either strain were added as indicated. Control IgG responses (no thymocytes added): TNP-KLH: 726 ± 53 PFC/10^6; TNP-Ficoll: 532 ± 91 PFC/10^6. Antigen doses were optimal: TNP-KLH 0.002 μg/ml; TNP-Ficoll 0.1 μg/ml.

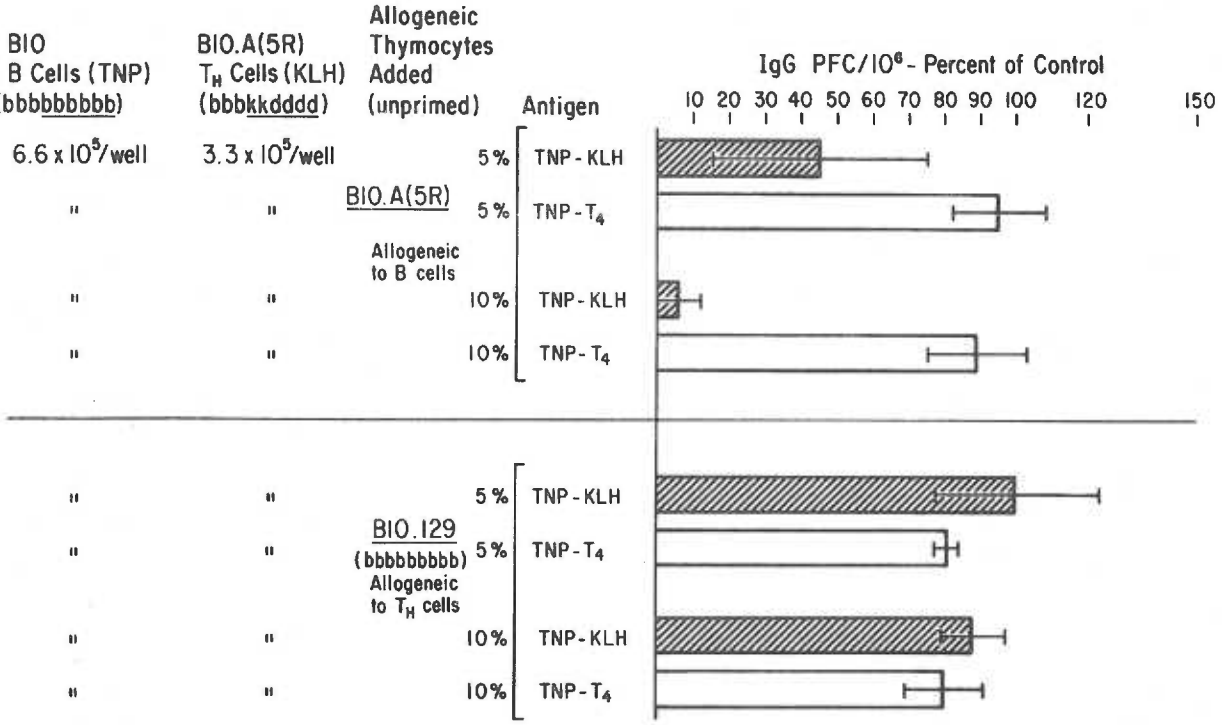

BIO.A(5R) B Cells (TNP) (bbbkkdddd)
BIO.A T_H Cells (KLH) (kkkkkdddd)
Allogeneic Thymocytes Added (unprimed)
Antigen
IgG PFC/10^6 - Percent of Control
10 20 30 40 50 60 70 80 90 100 120 / 180 / 240
6.6 x 10^5/well
3.3 x 10^5/well
BIO.A
Allogeneic to B cells
BIO.A(5R)
Allogeneic to T_H cells
5%
10%
TNP-KLH
TNP-Ficoll

the responding B cells. These thymocytes produced the same differential suppression of the TNP-KLH response observed in previous experiments (90% inhibition). In contrast, the TI response to TNP-Ficoll was not suppressed but rather was enhanced 2-fold. The addition of B10.A(5R) thymocytes, which were allogeneic only to the T_H cells, caused no significant change in either the TD or TI responses. Figure 4 shows the results of another such experiment in which the congenic strains used [B10 and B10.A(5R)] differ at the I-J, I-E/C, and D regions of the MHC. Once again, the differential allogeneic effects were observed only when the thymic effectors were allogeneic to the B cell and not when allogeneic to the T_H cells in the responding cultures.

In subsequent experiments we have studied the effects elicited by disparity at the I region [B10.A(4R) versus B10.A(2R)] or D region only [B10.A versus B10.A(2R)]. In all combinations used the same differential effects were observed. TD responses were inhibited and TI responses were unaffected or enhanced and in all cases only when the B cell was the target of the allogeneic thymocyte. In agreement with others (26) it was also noted that the allogeneic effects produced by I-region-only disparity were consistently of lower magnitude when compared to those elicited by K + I or D differences.

Discussion

The suggestion that murine B cells may be divided into two subpopulations differing in their requirement for T cell help as proposed by Playfair and Purves (29) has found support in a number of


BIO
B Cells (TNP)
(bbbbbbbbb)
BIO.A(5R)
T_H Cells (KLH)
(bbbkkdddd)
Allogeneic
Thymocytes
Added
(unprimed)
Antigen
IgG PFC/10^6 - Percent of Control
10 20 30 40 50 60 70 80 90 100 120 150
6.6 x 10^5/well
3.3 x 10^5/well
BIO.A(5R)
Allogeneic
to B cells
5%
TNP-KLH
TNP-T_4
10%
BIO.129
(bbbbbbbbb)
Allogeneic
to T_H cells

laboratories dealing principally with IgM responses (30-32), although in some instances IgG responses were included (3, 4, 33).

When we specifically analyzed memory B cells by several means including precursor analysis and independence of killing by BUdR and light after stimulation with TD or certain TI forms of the TNP hapten, it seemed clear that IgG-producing memory cells could also be divided into two responding populations designated B2γ and B1γ, respectively. Although B1 and B2 could represent distinct cell lineages (33, 34), it is more likely that they represent functionally different stages of a common lineage (35-37); however, since this question is by no means answered, the ability to distinguish these cells via differences recognized by allogeneic effector cells could constitute an important step in analyzing their relationship.

In the present study we have examined the ability of alloreactive T cells obtained from thymuses of unprimed young animals to modulate the expression of B1γ and B2γ memory cells. We found that these cells discriminated between these two memory populations; the secondary TD response to TNP-KLH was consistently suppressed. In contrast the TI responses to TNP-T_4 or TNP-Ficoll were enhanced under the same conditions (Table I). Maximum suppression was usually produced by 10% allogeneic thymocytes.

Although it was reported recently that alloreactive cells suppressed secondary responses to both TD and TI antigens (27), those results are not at variance with our data since in the former study

the response to the TI antigen was mainly of the IgM class and required approximately 10-fold more alloreactive cells for maximum suppression than did the TD response, thus also indicating a differential sensitivity of TI and TD secondary responses.

Our results are also in agreement with several *in vivo* studies in which F_1 mice undergoing mild graft-vs-host (GVH) reaction after administration of parental T cells, demonstrated prolonged suppression of a sheep red blood cell response, yet responded normally to LPS and showed an elevated response to the TI antigen polyvinylpyrrolidone (38, 39).

Limiting dilution analysis (Fig. 1, Table II) clearly demonstrates that B1Y and B2Y memory precursors may be directly affected by the allogeneic effector cells in a dose-dependent fashion and in opposite directions, as was also seen in standard microcultures; thus allogeneic effects do not result merely from altered burst sizes of the responding cells. However, before conclusions could be drawn concerning the difference between memory subsets, it was essential to confirm that in this system B cells were directly affected by both types of regulatory mechanisms. Using various congenic strains of the B10 series we were able to obtain cooperation between primed carrier-specific T cells and hapten-specific B cells with partial histoincompatibility (Fig. 2, Table III). Other investigators have previously reported successful collaboration across histocompatibility barriers after elimination of allosuppressors by various means, both *in vivo* (40) and *in vitro* (12, 15), although in one instance such success was limited to IgM memory cells lacking C receptors (41).

Using this model we were able to demonstrate that B cells are a direct target of alloreactive cells (Figs. 3 and 4). No significant changes in IgG responses were observed in cultures where thymocytes were allogeneic only to the T_H cells. Since B cells are important in triggering alloreactivity it should be noted that approximately 15% Ig^+ cells were present in the carrier-primed mitomycin C-treated target helper cells. Furthermore, Swain (27) has shown that mitomycin C-treated B cells can activate allosuppressors. Therefore, it seems unlikely that allogeneic T cells would not have been stimulated in this situation; since no effect was observed it is unlikely that the T helper cell is the target of the allosuppressor. These observations also argue against macrophages (MØ) being the major target of the alloregulatory cells since dysfunction of the MØ in the carrier primed population should have resulted in inadequate T_H stimulation and T-B signals. Similar conclusions were reached by others using a different approach to define the cellular targets of allogeneic T cells (12, 17).

Genetic differences in either the "left" or "right" portions of the H-2 complex were sufficient to stimulate the allogeneic effector cells in these experiments and in other experiments not shown. Furthermore, even when disparity was restricted to the I-region or the D-region, both allosuppressor and allohelper cells were stimulated, supporting other recent findings (13, 26).

What is the basis for the differential sensitivities of B1γ and B2γ to allogeneic effects? Although it is possible that H-2 encoded determinants are differentially expressed on B cell subpopulations, preliminary experiments treating primed lymphocytes with anti-Ia^k

antiserum and C resulted in elimination of both B1γ and B2γ precursors (unpublished observation) although quantitative differences are still possible. Another possibility, perhaps more likely, is that alloreactive cells, once stimulated, exert their effects via non H-2 encoded surface receptors, which are differentially expressed on B1γ and B2γ, similar to the restricted expression of some Lyb determinants (42-44). Alternatively, each type of T effector could recognize the same receptor on B1γ and B2γ, but the activation states of these subpopulations could differ such that B2γ only reacts in a negative manner whereas B1γ only reacts in a positive fashion under these experimental conditions.

A related question of major interest concerns the origin of the additional B1γ precursors that are activated by the allogeneic T cells. At least three possibilities exist as illustrated in Table IV.

Hypothesis a): Additional B1γ precursors could develop due to enhanced isotype switch of memory IgM to IgG-secreting cells. This would be in keeping with reports on the development of primary IgG responses to TI antigns if GVH reactions were initiated at the time of immunization (45, 56). However, since we are generally unable to detect many IgM PFC or their precursors in these cultures, this possibility appears unlikely.

Hypothesis b): The concomitant reduction in the numbers of B2γ precursors, which approximately paralleled the increase in B1γ precursors, could indicate that at least some of the newly arising B1γ precursors differentiated from B2 under the influence of the alloreactive cells. This hypothesis is strongly supported by the data in Table II;

TABLE IV

Hypothetical differentiation pathways leading to increased frequency of B1γ precursors in cultures subjected to allogeneic effect

Hypothesis	Early Precursor	Direct prescursor
a	B1μ	
b	B2γ	Allogeneic → B1γ → Antigen → B1γ PFC
c	B1γ preprogenitor	Signal Signal

however, the increase in Blγ was not always seen in standard microcultures, and when seen it occurred even after the negative effect had been eliminated by mitomycin C. Thus, it will be necessary to test this hypothesis more directly, perhaps by BUdR and light experiments.

Hypothesis c): Some precursors of Blγ may be arrested at an earlier stage of differentiation not able to respond under conventional culture conditions and are converted into antigen-responsive precursors after receiving a nonantigen-specific signal from the allogeneic cells. Such early precursors would be analogous to the memory preprogenitors proposed by Shortmann et al. (47, 48).

Regardless of the mechanisms or developmental pathways forming the basis of these obervations, one may propose that a population of memory B cells at a stage of development that is relatively free from thymic influences (either help or more importantly, suppression) would provide a means of maintaining a baseline immunologic memory independent of fluctuations in normal T cell regulatory controls.

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PAPER 3.

Differential Modulation of B Subpopulations in mice undergoing Graft-versus-Host Reaction (GVHR) and by *in vitro* induced allogeneic effects

Abstract

We have previously found, using an *in vitro* "allogeneic effect" model that secondary IgG responses to T-dependent (TD) and T-independent (TI) antigens were modified differently by alloreactive thymus cells. The memory TD response was very sensitive to negative "signals" whereas TI IgG responses were resistant to suppression and were frequently enhanced by the presence of allogeneic T cells (positive allogeneic effect).

In the present studies we examined the sensitivity of primary TD and TI responses to allogeneic effects induced *in vitro* or *in vivo* in F1 mice undergoing graft-versus-host reaction (GVHR).

It was found that primary TD responsive B cells (B2μ), similar to their memory IgG counterparts (B2γ) were very sensitive to negative allogeneic effects elicited *in vitro*. The primary TI responses (B1μ), on the other hand, were not suppressed but rather enhanced 2-3 fold under the same culture conditions.

When TNP-primed or unprimed F1 mice were immunized with TD (TNP-KLH, TNP-γ-sheep-globulin) or TI (TNP-Ficoll) antigens shortly after induction of GVHR, similar, opposite modulation of the *in vivo* PFC responses were observed. Both IgM and IgG primary responses to TD antigens were reduced while the *in vivo* primary response to TNP-Ficoll was enhanced and included (unlike untreated mice) a large number of primary IgG PFC indicating an extensive IgM to IgG switch. It was also found that mice undergoing chronic GVHR were incapable of developing long term memory cells following immunization with TNP-KLH as determined by their unresponsiveness to secondary *in vitro* challenges

with either TD or TI antigens. These mice retained normal levels of KLH-specific helper T cells thus localizing the defect in memory development to the B cell compartment.

These studies suggested that the secondary TI responding precursors (Bly) are probably derived from primary B2 precursors (which are very sensitive to negative allogeneic effects). The maturation of the B1 memory population is thus linked to increased resistance to allosuppression. Furthermore, a large portion of the memory pool is comprised of cells which can be driven into a TI antigen-sensitive state after receiving a non-antigen specific signal from alloreactive T cells.

Introduction

The data in Manuscript 2 showed that B subpopulations in the memory pool are differentially affected by allogeneic thymocytes. From an ontogenic point of view it was of interest to determine whether primary subpopulations would also be differentially affected by such nonantigen specific mechanisms. In early studies (by others) on allogeneic effects in primary cultures, the antigen most commonly used was sheep erythrocytes (SRBC). The response to this multi-determinant cellular immunogen was predominantly enhanced by unprimed allogeneic T cells (1-2), and it was suggested that in general, primary B cells benefit from allohelp even in the presence of allosuppressors while primed B cells are mainly suppressed under the same conditions (3). This view however, did not hold true in other studies which demonstrated that the primary SRBC response can be suppressed if mixed

lymphocyte reaction (MLR)-primed T cells are used as the allogeneic effector cells or if spleens of animals undergoing GVHR were used (4, 5). Furthermore, SRBC is a unique T dependent antigen in that unlike soluble TD antigens it can initiate B cell proliferation (but not differentiation) in the absence of T cells (6). In addition, it was suggested by the early in vivo studies of Playfair and Purves (7) and others (8) that the SRBC response has a TI component (at least in vivo).

Thus we decided to examine the regulation of primary responses by allogeneic T cells using the more defined TD and TI forms of the TNP hapten which have been used in our studies of secondary responses. We have also extended these studies to the in vivo induced allogeneic effects which take place in mice undergoing graft versus host reaction.

We found that the primary TD IgM response similar to its secondary IgG counterpart was sensitive to negative allogeneic effects elicited in vivo during GVHR or in vitro when antigenic stimulation takes place in the presence of unprimed allogeneic thymocytes. In contrast, primary TI responses were usually enhanced by the allogeneic effects indicating that the discriminatory effects of allogeneic cells may reflect an inherent difference between TD and TI responses.

Materials and Methods

Mice. BALB/c X C57BL/6 (CB6/F1), BALB/c X A/J (CA/F1), C57BL/6xDBA/2(B6D2F1) and C57BL/6 female mice were obtained from Jackson Laboratories, Bar Harbor, Maine. BALB/c breeders were from F. Hutchinson Cancer Center, Seattle, WA., and B10.A (2R) were purchased

from ARS Sprague-Dawley, Madison, WI. (CBA/N x BALB/c) F_1 females were from our breeding colony.

Antigens. TNP-KLH was produced by trinitrophenylation of KLH according to the method of Rittenberg and Amkraut (9) and had a mole ratio of $TNP_{1356}KLH$ based on a m.w. of 8×10^6 for KLH. TNP-sheep γ globulin, $TNP_{36}SGG$ was prepared as described by Scott (10). TNP-Brucella abortus (TNP-Ba) was produced according to the method of Mond et al. (11).

Immunization protocols. Mice were primed with two or three intraperitoneal injections of TNP-KLH-bentonite or KLH-bentonite (100 µg protein in 0.5 ml saline) every second week, as described before (12). In vivo primary responses were initiated by i.p. injection of TNP-Ficoll (100 µg in saline) or one of the two TD antigens; TNP-KLH and TNP-SGG. These antigens were each (100 µg/dose) mixed with an equal volume of alum (Maalox) and 10^9 killed Bordetella pertussis organisms (Eli Lilly and Co., Indianpolis, IN.) per dose. Spleens were assayed 5-7 days after injection. Three spleens were pooled and assayed in triplicate slides for each experimental point. Alternatively, in some experiments the spleens of 5 mice per group were assayed indivi- dually. Both direct (IgM) and indirect (IgG) PFC were measured (13-14).

Graft-versus-host reactions (GVHR). GVHR was induced by i.v. injection of $6\text{-}7.5 \times 10^7$ parental lymphocytes into F1 recipients via the tail vein. Donor lymphocytes were obtained from thymuses and spleens pooled together.

Allogeneic effects in cultures. Spleen cells from three mice primed with KLH-bentonite (or TNP-KLH on bentonite) were pooled and cultured as described in our previous paper (15) in the presence of various TD or TI antigens. Allogeneic or semiallogeneic thymocytes were added at the beginning of the cultures. In some experiments, mitomycin-C treated or untreated thymocytes were added so as to distinguish between effects due to suppressor T cells (mitomycin C-sensitive) and those mediated by helper T cells whose function is not reduced by such treatment (15-16). Cultures were assayed after 5 days (primary) or 7 days (secondary) using TNP-SRBC in a Cunningham slide assay (13).

Results

Allogeneic effects in primary and secondary cultures. In Table I, the effects of allogeneic (BALB/c, H-2^d) thymocytes on primary and secondary cultures of B10.A(2R) (H-2^h) lymphocytes are compared. As can be seen the primary TD response is 60% suppressed in the presence of 10% allogeneic thymocytes. Similarly the secondary IgG TD response was also suppressed by about 50% under the same conditions. If the BALB/c thymocytes are first treated with mitomycin C only about 20% suppression was detected which was not statistically significant ($P>0.1$). The TI responses, on the other hand, were enhanced in the presence of BALB/c thymocytes and even more so in the presence of mitomcyin-C treated thymocytes. Similar results were obtained in another strain combination (Table II). In these experiments, KLH-primed BALB/c lymphocytes were stimulated in vitro in the presence

TABLE I

The differential allogeneic effects of BALB/c (H-2^d) thymocytes on primary and secondary B10.A(2R) (H-2^h) *in vitro* PFC responses

		Type of Response			
		Primary		Secondary	
Antigen	Thymocytes added[a]	(IgM PFC/10^6)	P[b]	(IgG PFC/10^6)	P
TNP-KLH	None	413 ± 47[c]		1074 ± 30[d]	
TNP-KLH	10% BALB/c thymocytes	249 ± 35	P<0.05	520 ± 20	P<0.05
TNP-KLH	10% BALB/c thymocytes mitomycin C treated[e]	330 ± 18	P>0.1	840 ± 84	P>0.5
DNP-Dextran	None	200 ± 17		317 ± 62	
DNP-Dextran	10% BALB/c thymocytes	440 ± 87	P<0.05	450 ± 50	P<0.05
DNP-Dextran	10% BALB/c thymocytes mitomycin C treated	667 ± 42	P<0.01	800 ± 130	P<0.02

FOOTNOTES - Table I

[a]1×10^5 unprimed thymocytes were added to 1×10^6 KLH primed (primary response) or TNP-KLH primed (secondary response) B10.A(2R) spleen cells. Antigens were added at optimal dose: TNP-KLH 0.002 μg/ml; DNP-Dextran 0.01 μg/ml.

[b]P values were determined using student t test for non-paired data. Each experimental mean (response in presence of allogeneic thymocytes) was compared to the control response (no thymocytes added) to the same antigen.

[c]Mean ± standard error of triplicate cultures assayed on day 5.

[d]Mean ± standard error of triplicate cultures assayed on day 7.

[e]Thymocytes were treated with mitomycin C (25 μg/ml for 30 min at 37 C), washed three times and added to the responding cultures at the same cell ratio as above.

TABLE II

The differential allogeneic affects of normal C57BL/6 (H-2^b) thymocytes on primary *in vitro* responses of BALB/c (H-2^d) spleen cells

Thymocytes added	Mitomycin C treatment	Antigens added in culture (IgM PFC/10^6)					
		TNP-KLH (2.0 µg/ml)	TNP-KLH (0.2 µg/ml)	TNP-KLH (0.02 µg/ml)	DNP-Dextran (0.01 µg/ml)	TNP-Ficoll (0.001 µg/ml)	TNP-B. abortus (1:10^4) dilution)
BALB/c	-	229 ± 42 (100)[c]	279 ± 33 (100)	205 ± 75 (100)	171 ± 25 (100)	65 ± 15 (100)	696 ± 45 (100)
C57BL/6	-	105 ± 25 (45)	180 ± 34 (64)	64 ± 8 (31)	282 ± 29 (160)	256 ± 40 (340)	890 ± 72 (120)
C57BL/6	+	239 ± 112 (104)	232 ± 64 (92)	256 ± 75 (125)	378 ± 24 (220)	204 ± 22 (320)	950 ± 21 (136)

FOOTNOTES - Table II

[a] 1×10^5 unprimed syngeneic or C57BL/6 thymocytes were added to 1×10^6 BALB/c KLH-primed lymphocytes and cultured with antigens for 5 days.

[b] Mitomycin C treatment of C57BL/6 thymocytes was done as described in the legend to Table I.

[c] Numbers in parentheses are the percentages of the individual responses compared to the relevant control response to which syngeneic BALB/c thymocytes were added (100%).

of BALB/c (syngeneic) or C57BL/6 (allogeneic, H-2^b) thymocytes (or mitomycin C treated thymocytes). Three different doses of TNP-KLH were used to ensure that the suppression observed was not merely the result of a shift in the dose response to this antigen. As can be seen, all three doses gave comparable responses with a small peak at 0.2 μg/ml. More important, all three TNP-KLH responses were reduced (36-55%) in the presence of C57BL/6 thymocytes, but not in the presence of mitomycin C treated thymocytes. The TI-2 responses (to DNP-Dextran and TNP-Ficoll) were uniformally enhanced by the allogeneic thymocytes even without pretreatment with mitomycin C demonstrating the same resistance to allosuppression as their memory, TI-2 responding Blγ, counterparts. The response to TNP-B. abortus (TI-1 antigen) was significantly elevated over control ($P<0.05$) only in the presence of mitomycin C treated allogeneic thymocytes. This find- ing is probably due to the fact that this antigen can stimulate both Bl and B2 primary precursors [as was demonstrated previously in the memory response (17)] which are modified by the allogeneic effects in opposite fashion. Thus, we would attribute the enhancement of the TNP-Ba response, observed in the presence of mitomycin C treated allogeneic thymocytes, to augmentation of the Bl population. However this interpretation requires formal proof.

Another system which gives rise to allogeneic stimulation is the semiallogeneic parent→Fl combination. In this case T cells of parent A can recognize histocompatibility antigens of parent B which are expressed by all (AxB) Fl cells and may develop into effector cells

(e.g. helper, suppressors, killers) specific for the B parent haplotype of the F_1 recipient.

Table III contains the results of one such experiment. Unprimed BALB/c thymocytes ($H\text{-}2^d$) were added to primary cultures of (BALB/c X A/J)F_1 (CAF1,$H\text{-}2^{d/a}$) KLH primed spleen cells which were stimulated with TNP-KLH or various TI forms of TNP. It was found again that the TI responses to TNP-Ficoll and DNP-Dextran were enhanced 2-3 fold over the control cultures. The TNP-KLH response was suppressed by 59% in agreement with the findings of the previous tables. The TI-1 response to the TNP-Ba was also enhanced 5-fold in this strain combination.

Modulation of in vivo primary responses by graft-versus-host reactions (GVHR). The in vivo parallel of the experiment presented in Table III is the graft-versus-host reaction which can be elicited by administration of parental lymphocytes into F1 recipients. In order to determine whether in vivo TD and TI responses also show differential sensitivities to allogeneic effects we immunized CAF1 ($H\text{-}2^{d/a}$) and CB6F_1 ($H\text{-}2^{d/b}$) (Table IV) or B6D2F1($H\text{-}2^{b/d}$) mice (Table V) with 100 g of TNP-SGG, TNP-KLH or TNP-Ficoll intraperitoneally shortly after intravenous administration of BALB/c (Table IV) or C57BL/6 (Table V) parental lymphocytes via the tail vein. It is important to emphasize that in early experiments it was found that F1 animals inoculated with syngeneic lymphocytes at the time of immunization gave comparable responses to those of animals not receiving any cells before immunization; (nevertheless, in some experiments control animals were inoculated with syngeneic lymphocytes (see Table VI).

TABLE III

In vitro allogeneic effects in primary responses of CAF1 mice mediated by parental (BALB/c) thymocytes[a]

Thymocytes added	Antigen in culture (IgM PFC/10^6)			
	TNP-KLH	TNP-Ficoll	DNP-Dextran	TNP-B. abortus
-	111 ± 22	76 ± 21	210 ± 54	206 ± 69
10% BALB/c thymocytes	46 ± 11	226 ± 90	475 ± 56	1147 ± 119

[a]KLH-primed CAF1 spleen cells were cultured with optimal antigen doses: (TNP-KLH 0.02 µg/ml TNP-Ficoll 10^{-3} µg/ml; DNP-Dextran 0.01 µg/ml; and TNP-B. abortus 1:10^4 dilution) in the absence or presence of 10% BALB/c thymocytes (unprimed). All cultures were assayed on day 5.

TABLE IV

Modulation of *in vivo* primary responses of CB6/F1 and CAF1 mice by GVHR induced with BALB/c lymphocytes at the time of immunization

Exp. No.	Responding Strain	Antigen	GVH[a]	IgM PFC/10^6	IgG PFC/10^6
1	CB6F1	TNP-KLH[b]	-	182 ± 18[c]	534 ± 32
			+	31 ± 21 (17%)[d]	341 ± 17 (63%)
		TNP-Ficoll[e]	-	150 ± 6	20 ± 2
			+	124 ± 6 (83%)	493 ± 32 (2400%)
2	CAF1	TNP-SGG[b]	-	212 ± 6	398 ± 27
			+	39 ± 3 (18%)	117 ± 2 (29%)
		TNP-Ficoll	-	572 ± 24	34 ± 2
			+	1325 ± 87 (230%)	407 ± 23 (1200%)
			+ (mitomycin C treated)[f]	562 ± 36 (98%)	71 ± 8 (200%)

[a]Graft-versus host reactions (GVH) were induced by administration of 6-7.5 x 10^7 BALB/c lymphocytes into F1 recipients via the tail vein shortly before antigen administration.

[b]TNP_{36}SGG and TNP_{936}-KLH were each mixed with an equal volume of alum and 10^9 *Bordetella pertussis* organisms. Each animal (3 per group) was injected i.p. with one dose containing 100 µg of the relevant antigen. Administration

FOOTNOTES - Table IV (Continued)

of alum and Bordetella pertussis alone did not elicit significant numbers of IgM or IgG TNP specific PFC (data not shown). Spleens were assayed 7 days post immunization.

[c]Mean ± standard error of 3 slides made from a spleen cell pool of 2-3 mice, 5 or 7 days after antigen administration (see Results section).

[d]The numbers in brackets represent the response of the GVH group as a percentage of the response of the control (untreated) group.

[e]TNP-Ficoll was solubilized in saline. Each mouse received 100 µg i.p. Spleens were assayed 5 days (Exp. 3) or 7 days (Exp. 1, 2) post immunization.

[f]Parental cells were treated with mitomycin C (25 µg/1 x 10^7 cells) and washed extensively before administration into F1 recipients.

Four representative experiments (out of 8) are presented in Tables IV and V. It can be seen that in all three mouse strains, the in vivo induced allogeneic mechanisms (in the form of GVHR) led to suppression of the TD responses (to TNP-SGG and TNP-KLH). Both IgM and IgG primary responses were suppressed (80-91% and 37-82% inhibition respectively). The data in Table V were calculated both per 10^6 spleen cells and per spleen (each experimental group included five mice which were assayed individually), in order to establish that the suppression observed was not merely a reflection of "dilution" of the TNP-specific precursors due to proliferation of other unrelated cells (of donor or host origin) which usually takes place in GVH spleens. As can be seen, in either way of presentation, the calculated inhibitions of the TD responses by the allogeneic effector cells were of similar magnitudes. The TI response to TNP-Ficoll was modified in a completely opposite fashion. First, it should be noted that unprimed mice when stimulated in vivo or in vitro with TNP-Ficoll (and most other TI antigens), produce antibodies which are mainly of the IgM isotype. On the other hand, mice which received parental lymphocytes shortly before antigen inoculation not only gave enhanced IgM responses (Table IV exp. 2, Table V exps. 1 and 2) but also developed a large IgG primary response (12-26 fold increase over control groups). These data suggest therefore that under GVH conditions both recruitment of additional B1 precursors and enhanced IgM to IgG switch occur simultaneously in the responding clones. We have also tried to separate the positive and negative effector mechanisms by in vitro treatment of the parental cells with mitomycin

TABLE V

Modulation of *in vivo* primary responses of B6D2 F1 mice by parental (C57BL/6) lymphocytes. Enhancement and suppression are both sensitive to treatment of donor cells with mitomycin C.

Exp. No.	Antigen *in vivo*	Cells administered *in vivo*[a]	Mitomycin C treatment[b]	PFC/10^6 IgM		PFC/10^6 IgG		PFC/spleen ($\times 10^{-3}$) IgM		PFC/spleen ($\times 10^{-3}$) IgG	
1	TNP-SGG[c]	F1 → F1	-	191±12[d]	(100)	2295±441	(100)	17±2	(100)	159±30	(100)
		C57BL/6 → F1	-	34±12	(18)[e]	462±69	(20)	7±2	(38)	64±9	(40)
	TNP-KLH[c]	F1 → F1	-	304±70	(100)	2529±254	(100)	28±6	(100)	258±25	(100)
		C57BL/6 → F1	-	60±16	(20)	892±138	(35)	11±3	(37)	116±37	(45)
	TNP-Ficoll[f]	F1 → F1	-	1478±98	(100)	80±2	(100)	127±6	(100)	10±1	(100)
		C57Bl/6 → F1	-	2356±172	(160)	1834±234	(2300)	226±10	(180)	120±8	(1220)
2	TNP-SGG	F1 → F1	-	195±20	(100)	1176±146	(100)	34±3	(100)	165±36	(100)
		C57BL/6 → F1	-	17±4	(9)	217±30	(18)	5±1	(16)	35±7	(21)
		C57Bl/6 → F1	+	150±23	(77)	1348±120	(114)	28±4	(83)	153±12	(93)

TABLE 5 - Continued

TNP-Ficoll	F1 → F1	-	1189±66	(100)	52±11	(100)	79±9	(100)	4±1	(100)
	C57BL/6 → F1	-	2754±37	(230)	1654±110	(3180)	271±28	(340)	162±6	(3600)
	C57BL/6 → F1	+	921±132	(79)	69±13	(130)	75±10	(95)	6±1	(140)

[a]B6D2 F1 mice received 7.5×10^7 syngeneic (control group) or parental (C57BL/6) lymphocytes via the tail vein shortly before intraperitoneal administration of antigen.

[b]Donor cells were treated with mitomycin C (25 µg/10^7 cells) as described in Table I.

[c]TNP_{36}SGG and TNP_{936}-KLH were mixed with alum and pertussis (as in Table IV). Each animal received 0.2 ml containing 100 µg of the relevant antigen. Spleens were assayed individually 7 days later.

[d]The data are presented as mean ± standard error of 5 spleens assayed individually in each experimental group.

[e]The numbers in parentheses represent the responses of the GVH group as a percentage of the response of the control (F1 → F1) group.

[f]Soluble TNP-Ficoll was given i.p. (100 µg/0.1 ml of saline). Spleens were assayed individually 5 days later.

C before administration into the F1 recipients [similar approach was successful in dissecting the in vitro induced allogeneic effects, Tables I, II and (15)]. It was found (Tables IV, VI) that mitomycin-C treated donor cells were incapable not only of inducing splenomegaly in the recipient mice, but also of generating both inhibitory and enhancing effector cells. We must conclude in agreement with others (18) that donor's alloreactive precursors must undergo at least several rounds of proliferation after transfer into F1 recipients before differentiating into effector cells (helpers, supressors, etc.)

Modulation of in vivo memory expression by GVHR. Thus far we have shown that in vivo or in vitro primary TI and TD responses can be differentially modulated by allogeneic effects. We have also shown (15 and Table I), that in vitro secondary IgG PFC responses are similarly modulated. The in vivo secondary responses of TNP-primed (CBA/N X BALB/c) F1 females or (BALB/c X C57BL/6) CB6 F1 mice and the effects of GVHR induced at the time of secondary challenge are depicted in Table VI. It was found that in vivo secondary IgG responses are modified by allogeneic effects in the same way (although in these experiments not to the same extent) as the in vitro secondary responses (15). In both cases the TD response was sensitive to the negative signals delivered by allosuppressors (35% reduction $p<0.02$) while the TI IgG response to TNP-Ficoll was amplified.

The effect of GVHR on induction of memory subpopulations. The data presented thus far, suggest that B subsets responding to TD and TI-2 antigens show differential sensitivies to allogeneic regulatory mechanisms. The ontogenic relationship between these subsets in the

TABLE VI

Differential modulation of *in vivo* secondary responses of F1 mice by GVH reactions induced at the time of antigenic challenge

Exp. No.	Strain	GVH[a]	Secondary challenge *in vivo* (IgG PFC/10^6) TNP-KLH[b]	p	TNP-Ficoll[b]	p
1	(CBA/NXBALB/c) F1 females	-	7192 ± 909		135 ± 19	
		+	4948 ±1015 (65)[c]	<0.02	1638 ± 43 (1200)	<0.001
2	CB6/F1	-	2306 ± 118		147 ± 12	
		+	1500 ± 82 (65)		10,000 ± 755 (6800)	<0.001
		+ (Mitomycin C treated)[d]	2781 ± 147 (120)	>0.5	ND[e]	

[a]Graft-versus-host reactions (GVHR) were induced by administration of 6-7.5 x 10^7 BALB/c lymphocytes into F1 recipients via the tail vein shortly before antigen administration.

[b]TNP-KLH or TNP-Ficoll were dissolved in saline and 100 µg were injected i.p. in 0.1 ml volume. Spleens were assayed 5-6 days later (pool of 3 spleens per experimental group).

FOOTNOTES - Table VI (Continued)

[c]The numbers in parentheses represent the responses of the GVH group as a percentage of the response of the control (untreated) group.

[d]Mitomycin C treatment of BALB/c donor cells was conducted as described in the legend to Table I.

[e]ND; not done.

primary and secondary pools has not been determined yet. However, we do know that vigorous IgG memory responses to both TD and TI antigens can only be induced if TD antigen is used as the priming antigen (19-20). Since, in our system, primary TD responses are sensitive to allosuppression while TI responses are not, it appeared possible to immunize mice with a TD antigen while they were undergoing GVH and to follow memory development among the subsets in order to determine whether they are similarly or differentially affected. Such an experiment could shed light on the ontogenic relationship between B1 and B2 memory populations.

In order to maximize allosuppression CB6F1 mice received three injections of BALB/c or C57BL/6 parental lymphocytes; 14 and 7 days before the first antigen dose and a third injection of cells 7 days before the second priming dose. Control groups were injected with syngeneic F1 lymphocytes according to the same protocol. The data in Table VII represent the results of these experiments. As anticipated, no memory B2γ cells could be detected in the GVH group indicating that suppression of the primary precursors interfered not only with the development of primary B2 PFC response, but also resulted in abberant B2γ memory propagation. We could not predict, however, whether memory B1γ precursors would develop normally or not. If B1γ developed normally in the absence of B2γ memory cells it would suggest independent rather than linked development of the two B memory pools. As can be seen in Table VII, GVH reactions almost completely suppressed ($\geq$87%) the development of both TI-1 (TNP-Ba) and TI-2 (DNP-Dextran) secondary responses. The same picture was obtained on day 5 of culture (not

TABLE VII

GVH reactions prevent the development of B2γ and B1γ memory populations following TNP-KLH priming of CB6/F1 mice[a]

	Antigen in secondary culture[c]		
GVH Induction[b]	TNP-KLH (IgG PFC/10^6)	DNP-Dextran (IgG PFC/10^6)	TNP-B. abortus (IgG PFC/10^6)
-	573 ± 93	331 ± 40	1100 ± 95
F1 → F1	500 ± 87	330 ± 52	800 ± 49
C57B1/6 → F1	0 ± (0%)[d]	0 ± (0%)	100 ± 34 (13%)
BALB/c → F1	14 ± 16 (3%)	24 ± 8 (8%)	100 ± 64 (13%)

[a]The priming antigen, TNP-KLH on Bentonite was given in two doses containing 100 μg TNP-KLH each. The first dose was given i.p. after 2 injections of parental cells (a week apart) and the second antigenic dose, 7 days after the third injection of parental cells.

[b]Graft-versus-host reactions (GVHR) were induced by 3 injections of syngeneic (F1) or parental (C57BL/6 or BALB/c) lymphocytes. 6.5×10^7 cells per dose were injected via the tail vein.

FOOTNOTES - Table VII (Continued)

[c]Mice were rested for at least 2 months before sacrifice. Spleens of 2-3 animals were pooled and cultured with the various antigens at optimal antigenic doses (TNP-KLH, 0.002 μg/ml; DNP-Dextran 0.01 μg/ml; TNP-B. abortus $1:10^4$ dilution). The data represent day 7 assay. Similar results were obtained on day 5 of culture.

[d]Mean ± standard errors of triplicate cultures. Numbers in parentheses represent percentages of the control (F1 → F1) responses.

shown), and both parental strains were equally effective in abrogating memory development in the F1 recipients.

Are helper T cells affected in mice undergoing GVHR? Since functional T cells are essential for TD responses and for propagation of IgG memory (20-21), it was important to assess the level of T-helper (TH) activity in mice undergoing GVH disease.

Spleen cells from GVHR (C57BL/6 → CB6/F1) mice were thus irradiated by 1200 rads from a cesium source (this dose is sufficient to kill B cells and T suppressor cells but not helper T cells) (16, 22-24), and were then combined with TNP-primed B cells (anti thy 1.2+c' treated spleen cells) from control mice which received F1 syngeneic cells during the immunization protocol (the mice used in this experiment were from the same groups presented in Table VII). Table VIII summarizes the results of this experiment. Anti thy 1.2+c' treatment reduced the TNP-KLH response by 96%. The TI response to TNP-Ba was only slightly reduced (17%, $p>0.1$). When irradiated spleen cells from TKB primed and GVH treated mice were combined with the T-depleted B cells, the response to TNP-KLH was reconstituted to about 75% of control ($p>0.5$) while the TNP-Ba response was reduced by 50%. This reduction can be easily explained, since the number of B cells per culture was reduced by half. It was thus concluded that the mice undergoing GVHR do retain considerable if not normal numbers of functional T-helper cells suggesting therefore that the failure of specific memory to develop in mice undergoing GVH disease is due to direct effects on the memory precursors themselves.

TABLE VIII

T helper cells are not affected in mice undergoing chronic GVH disease

Responding spleen cells: $CB6F_1$ injected with $CB6F_1$ lymphocytes and TNP-KLH primed	Anti thy 1.2 +C' treatment[a]	T-helper cells:[b] from $GVHF_1$ mice ($C57BL/6 \rightarrow CB6F_1$)	TNP-KLH IgG PFC/10^6	TNP-B. abortus IgG PFC/10^6
10×10^5/well	-	-	210 ± 80	580 ± 41
10×10^5/well	+	-	18 ± 12 ($p<0.001$)	480 ± 26 ($p>0.1$)
5×10^5/well	+	5×10^5/well	141 ± 22 ($p>0.5$)	240 ± 49 ($p<0.01$)

[a]Spleen cells were treated with monoclonal anti-thy 1.2 antibodies (HO-13-4) (30) and complement as described in (31). Red cells were removed by ammonium chloride treatment before anti-thy treatment. The control cells were treated similarly substituting MEM for anti-thy antiserum.

[b]Spleen cells were obtained from $CB6/F_1$ mice which had been primed with TNP-KLH during the course of a GVH disease. Cells were irradiated with 1200 rads from a cesium source and mixed at a 1:1 ratio with anti-thy 1.2 + C' treated, TNP primed B cells.

Discussion

The data presented in this paper extend our previous studies on the differential sensitivites of B cell subpopulations to nonantigen specific regulatory mechanisms mediated by allogeneic thymocytes (15). It was found that primary in vitro IgM responses can be modified by allogeneic or semiallogeneic (P→F1) thymocytes in a similar way to the in vitro secondary IgG responses (15). Addition of 10% allogeneic thymocytes at the initiation of culture resulted in partial inhibition of the TNP-KLH response and enhancement of the TI responses (DNP-Dextran, TNP-Ficoll and TNP-T4) (Tables I-III). Treatment of the allogeneic thymocytes with mitomycin C eliminated the suppression of the TD response, but did not interfere with the enhancement of the TI responses. The response to soluble TD antigens (such as TNP-KLH) therefore appears to be fundamentally different from the response to the cellular TD immunogen SRBC which can be significantly enhanced by small numbers of allogeneic thymocytes (2-3, 16). Keller et al. (6) have recently shown that the requirements for activation by soluble T-dependent hapten-carrier conjugates are much more complex compared to cellular antigens in that T cell depleted B cells can be triggered by SRBC if T cell replacing factor (TRF) is added as late as 72 hours after the antigen (25). No activation by TNP-KLH took place under such conditions unless additional, antigen-specific T cells or factors were introduced together with the antigen (6). It is thus possible that cellular antigens such as SRBC modulate primary B2 precursors and convert them into a new physiological state more sensitive to non-specific signals such as provided by TRF and positive allogeneic

effect. The seemingly selective sensitivity of TI responding B cells to positive allogeneic effects may reflect a difference in their physiological state in unprimed (or primed) animals. The in vivo experiments depicted in Tables IV, V and VI gave a similar picture to that obtained in the in vitro cultures. Both primary and secondary in vivo TD responses (TNP-KLH, TNP-SGG) were reduced in mice undergoing GVHR. This inhibition could not be explained by suppression of the host T helper cells since irradiated GVH spleens (which lacked functional B cells) were able to reconstitute the TNP-KLH response of T-depleted, TNP-primed B cells (Table VIII). This conclusion is also in line with the study of Lapp et al. (26) who found that the number of thymus derived cells of F1 origin were not diminished in thymus, spleen or lymph nodes of animals experiencing GVH disease. Our results concerning the effects of GVH disease on in vivo TD primary responses are also in agreement with those of Osborne and Katz (27) and Feldmann and Basten (28) who were not able to boost in vivo primary responses of F1 mice to soluble TD antigens by concommitant administration of antigen and parental cells. In one report, by Klaus and McMichael (29), enhancement of primary TNP-KLH responses was observed. However, the response of the control mice was not optimal since no adjuvant was used and the mice were not KLH primed. It is not possible to say therefore that B2 populations can never benefit from allohelp and that B1 cells are absolutely resistant to allosuppression. Instead, we can safely state that under the conditions used in our experiments, the two subpopulations were modified differently by the allogeneic effects allowing the importance conclusion that

these B cells subsets possess distinguishing characteristics which can be recognized differentially. The above results also suggested that this model could be used as a tool to study the ontogenic relationship between B1 and B2 during the development of memory. It was found that induction of GVH during immunization of mice with TNP KLH resulted in virtually complete suppression of anti-TNP memory cell development, not only of the B2 but also of the B1 subset. The most simple explanation is that the B2 precursors which are sensitive to negative allogeneic effects are the ones giving rise to both B2 and B1 memory cells in normal animals primed by a TD antigen without interference. Since the development of B1 memory is suppressed by GVH while the expression of B1 memory seems refractory to negative allogeneic effects and most commonly is enhanced by GVHR or by allogeneic thymocytes in vitro, these cells must undergo a sharp developmental transition sometime after the priming events. It is hoped that if GVHD is induced at various time intervals after completion of the immunization protocol we would be able to identify this transition point.

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SUMMARY AND DISCUSSION

Previous studies in our laboratory and others (1-3) have established that the antigen responsive B cell pool in unprimed or primed animals contains functionally distinct subsets of cells which differ in their activation requirements (TD vs. TI). The studies in this dissertation dealt primarily with the interactions between these B cell subsets and regulatory effector cells using "allogeneic effects" as the model system. This term has been used to describe activation of T cells of different functional capabilities by histoincompatible B cells (4-5). It was argued by other investigators in this area that these regulatory mechanisms probably reflect at least some of the T-B interactions which take place in normal animals and determine the overall immune status of the individual (6-7).

This model system allowed us to go one step further towards understanding the physiological differences between B subpopulations and the types of signals leading to their activation or suppression. Furthermore, the data suggest that this system (in combination with other experimental approaches) will help in determination of the ontogenic relationships between the various subsets.

In this section I shall summarize the principal findings of this project and indicate some of the questions and the experimental approaches which can be taken in the future.

I. Summary of principal findings:

a. Primary TD (B2μ) and TI (B1μ) TNP-specific subpopulations were differentially modulated by allogeneic thymocytes in vitro. The response of B2μ cells was usually suppressed, while that of the B1μ population was enhanced.

b. Similar differential sensitivities to allogeneic effects were manifested in secondary cultures, of memory B2γ and B1γ populations. Limiting dilution analyses showed that the added allogeneic thymocytes caused simultaneous reduction of TD and increase of TI precursor frequencies.

c. Mitomycin C treatment of the allogeneic thymocytes eliminated the subsequent suppression of the TD responses (primary or secondary) but did not remove the enhancement of the TI responses indicating, in agreement with others (8) who have studied allogeneic help and suppression that they are induced by separate effector mechanisms.

d. Similar differential allogeneic effects were seen in vivo when F1 mice were injected intravenously with parental lymphocytes (GVH induction) at the time of primary or secondary antigenic challenge.

e. B cells are probably the direct targets of the alloreactive T cells, since mice primed with TNP-KLH while undergoing GVH disease retained normal levels of KLH specific TH cells while giving reduced TD antibody responses. Also, in in vitro secondary cultures in which the added thymocytes were allogeneic either to the B cells or TH cells it was found that recognition of the B cells alone resulted in supression of the TD responses and enhancement of the TI responses.

f. Priming of F1 mice with a TD antigen during an ongoing GVH reaction resulted in aberrant development of memory cells belonging to both the B1γ or B2γ subpopulations. These findings suggest that common primary precursors (most likely of the B2 lineage) give rise to both memory subpopulations under normal priming conditions.

II. Discussion of major findings and future studies

One of the points which emerged from this project was that B1 populations, while capable of responding to antigenic stimulation in the absence of carrier-specific T cells, can benefit from non-antigen specific T cell mediated help. Other studies have shown that responses to TI antigens can be modified by regulatory T cells. For example, the response to PVP could be inhibited by both antigen specific and nonspecific T suppressor cells (9) and the magnitude of the polyclonal response to LPS is also controlled by T cells(10). Recently it was found by Mond et al. (11) that the *in vitro* primary response to TNP-Ficoll can be markedly reduced by vigorous depletion of T lymphocytes suggesting at least a partial requirement for T cells for optimal responses to this TI-2 antigen. Also the *in vivo* response to SIII can be either amplified or suppressed by concanavalin A induced effector T cells (12-13). Other types of T mediated regulation of TI responses have also been described. Mongini et al. ( ) found that heterozygous nu/+ mice (which contain normal levels of T cell function) produced substantially larger amounts of antibodies of the IgG2 subclasses following immunization with TNP-Ficoll or TNP-Levan than their T-cell deficient, athymic (nu/nu) counterparts which

produced antibodies mainly of the IgM, IgG3 and, to a lesser extent, IgG1 classes. It was postulated that class specific T cells can influence heavy chain switching in TI-responding B cell clones. The same investigators also noticed that in addition to hastening the production of IgG after TNP-Ficoll immunization, the presence of T cells also caused a more pronounced fall in antibody titer after peak production was reached, and suggested that this might be the result of T-cell dependent anti-idiotype-induced regulation of the TNP-Ficoll responses, as was described by Schrater et al. (15). Also, in a recent paper by Takatsu et al. (16) it was shown that purified T cell replacing factor (TRF) produced by a T cell hybridoma was capable of enhancing secondary DNP-Dextran IgG responses in T-depleted spleen cells similar to the enhancing allogeneic effects observed in our system.

What are the mechanisms involved in these enhancing effects? Are they simply increasing the proliferative response of the B1 precursors following antigenic stimulation or do they recruit new TI precursors? Our limiting dilutions analyses of secondary cultures showed that allogeneic thymocytes caused a 4-fold increase in the frequency of TI IgG precursors (B1γ), although an enhanced proliferative response was not excluded as occurring at the same time. We are left with the question: what is the source of these additional precursors? Since the numbers of IgM PFC are usually very low in TNP-primed spleen cells, a simple B1$\mu \rightarrow$B1γ switch is not very likely to be the main source of additional precursors. Also, in primary responses we found that GVHR caused concomitant enhancement of both IgM and IgG TI PFC

responses again arguing that the additional IgG precursors were not derived as a result of an IgM G switch. The other two alteratives are: a. Bl precursors may be derived from B2 cells under the influence of the allogeneic cells, and b. The Bl cell pool contains not only antigen responsive cells but also significant numers of antigen insensitive precursor cells (or preprogenitors (17) which may undergo differentiation and acquire antigen sensitivity after receiving signals delivered by accessory cells which, our data suggest, are either T cells or other cells which interact with T cells or T cell products.

A second question raised by these results is whether the additional precursors represent the same heterogenous population of antibody forming cells as those detectable by antigen stimulation alone or if they respresent a more restricted population of higher or lower avidity.

Another point of interest is whether the same genetic loci are involved in allogeneic suppression of the B2 precursors and enhancement of the Bl precursors. This question has been partially approached during the experiments described in Manuscript 2. However a more detailed genetic analysis needs to be conducted using mouse strains which are congenic for genes in the major histocompatibility complex (MHC) or at minor histocompatibility loci such as the Mls locus.

It is hoped that once the goals described above are met, they will enhance our understanding of the various cell lineages which mediate humoral responses to haptens on different carrier molecules in terms of their activation requirements and lead to a better understanding of the ontogenic relationship between the primary and memory pools.

Such studies will also increase our insight into the mechanisms by which graft-versus-host reactions modulate host immune responses and sometimes lead to both undesired activation of auto-reactive clones and to supression of pathogen-recognizing cells. A better understanding of these phenomena could lead to discovery of a means of modifying grafted bone-marrow cells so as to eliminate or reduce these harmful effects.

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