ABSTRACT

REGULATION OF ANTIBODY RESPONSE BY
DNP CARRIER-PRIMED LYMPHOCYTES

By

Erich F. Eipert

Dinitrochlorobenzene (DNCB) produces antigen specific contact sensitivity in mice. It was previously shown that pretreatment of the animals with dinitrobenzene sulfonate (DNBS) renders them tolerant to subsequent DNCB sensitization. In the present study, DNCB skin sensitization did not induce spleen cells capable of transferring helper activity to mice challenged with DNP-sheep erythrocytes (SRBC) upon assay for anti-SRBC response. However, cells from mice treated with 15 mg of DNBS did exhibit a suppressive effect in such a system since direct and indirect anti-SRBC plaque forming cell responses were consistently lower than those of control groups receiving normal cells. A similar level of suppression was also evident in a secondary response. DNBS induced suppression appears to be antigen specific because suppression of the response did not occur when SRBC were substituted for DNP-SRBC as the challenge antigen. Suppression routinely achieved by the transfer of 1-2 x $10^7$ cells did not improve by transfer of $10^8$ cells and declined with the transfer of $10^6$ cells.

This inhibition of both the primary and secondary response could be at least partially abrogated by treatment of donor cells with rabbit anti-BA-Thy 1 serum prior to transfer. In this system it appears that suppressor cells are directly induced by DNBS rather than recruited from the recipient population because B cell reconstituted X-irradiated recipients given DNBS educated thymocytes or spleen cells showed a level of response inhibition similar to that seen in normal (nonirradiated) recipients given DNBS primed spleen cells. The relative level of suppression apparent upon comparison of responses in recipients of normal or DNBS primed cells was preserved when the responses of both groups were boosted by allogeneic enhancement. C3H parental as well as the C57/Bl x C3H $F_1$ cells used in the preceding experiments were receptive to both the suppressive and allogeneic signal, but only in the simultaneous presence of both $F_1$ and C3H parental type T cells. Responses of normal or x-irradiated B cell repopulated $F_1$ recipients given only DNBS primed parental spleen cells or T cells were allogeneically enhanced but not suppressed.

# REGULATION OF ANTIBODY RESPONSE BY DNP CARRIER-PRIMED LYMPHOCYTES

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# INTRODUCTION

*These are the four that are never content: that have never*
*been filled since the dews began--*
*Jacala's mouth, and the glut of the kite, and the hands of the*
*ape, and the eyes of man*

"The King's Ankus"
*The Jungle Books*
*Rudyard Kipling*

The case for dichotomy of the immune system, with B cells responsible for humoral immunity and T cells for cellular immunity, has perhaps been overstated in the past. This simplified scheme is currently challenged by evidence which suggests that the two types of immune responses are intimately connected through the participating cells. The nature of the relationship seems to be one of modulation whereby both T and B cells individually regulate certain activities of the other. The research reported in this communication has as its objective a mechanistic understanding of one particular interaction--T cell synergy with B cells in the regulation of antibody response. Such an insight of the events in this particular aspect of immune regulation is of prime importance in explaining the role of the immune system in cancer, autoimmunity, aging and atopy.

The simplistic notion that cooperation with B cells occurs on a superficial level and can be explained solely in terms of antigen

presentation by T cells is no longer tenable. Although our current comprehension of T cell involvement in antibody response is recognized to be incomplete, this involvement now seems to center about maintaining this response within reasonable limits. This level of apprehension, rudimentary as it may be, is the culmination of intensive research which has given us, in addition to any practical benefits, a perspective of the true complexity within this system.

The review portion of this communication describes those experiments in hypothetical models which had profound influences on development of the T cell regulatory concept. Through work carried out largely in the mouse system, these principles evolved from a number of diverse observations concerning T cells functions. The more notable of these findings demonstrated that T cells: 1) produce soluble factors in the mediation of cellular type responses, 2) like B cells are subject to antigen induced unresponsiveness, 3) are induced to develop memory upon antigen exposure, 4) exhibit an antigen recognition capacity comparable to B cells, and 5) are at least partly under the control of immune response (IR) genes located in the major histocompatibility gene complex. It is hoped that through the following review the significance and relatedness of these events will become as apparent as the need for further investigation.

The remainder of this communication consists of two manuscripts describing our experimental approach to understanding T cell control over antibody production by B cells by using a unique antigen-induced *in vivo* suppressor system. The findings have proven to be

compatible with the recent findings of others and are discussed in terms of possible mechanisms of action.

# LITERATURE REVIEW

Current concepts of T cell involvement in antibody response are based on research carried out by a great number of investigators working at both the cellular and molecular level and using a number of original approaches. The ensuing complexity has necessitated the presentation of this review in the following progression of concepts.

## Recognition of T - B Cooperation

The experiments of Claman *et al.* (1) were among the first to suggest a requirement for both T and B lymphocytes in an antibody response. Irradiated mice, given both thymus and bone marrow cells, made quantitatively better antibody responses to test antigen than did mice given either of the lymphocyte classes alone. Shortly thereafter, Davies *et al.* (2) in a more elaborate system showed that thymus derived cells were not antibody producing cells. Thymectomized and irradiated mice reconstituted with syngeneic bone marrow were further given allogeneic thymus cells and challenged with sheep red blood cells (SRBC). When spleen cells were transferred to recipients immunized against either the marrow or thymus alloantigen, antibody production was prevented only if the immunity was directed against the marrow cells. Miller and Mitchell (3) subsequently found that neonatally thymectomized mice, normally unable to produce antibody, are restored to producer status by adoptive transfer of

semiallogeneic thoracic duct or thymic lymphocytes. By the use of alloantisera they demonstrated that antibody producing cells were of host origin. Nossal *et al.* (4) confirmed these findings by showing that in irradiated recipients given chromosomally marked marrow cells and unmarked thymus cells all the antibody forming cells were of the chromosomally marked variety.

## Carrier Cell Involvement in T - B Cooperation

### Development of the Carrier Concept

Landsteiner (5) showed that hapten-specific antibody is induced when hapten is complexed to immunogenic carrier. Hapten-carrier conjugates have since become an important tool in immunology and have played a major part in clarifying T cell participation in humoral responses. Ovary and Benaceraf (6) used conjugates to demonstrate what has become known as the "carrier effect." They found that rabbits primed with dinitrophenylated bovine gamma globulin (DNP-BGG) produced a secondary response to DNP if the secondary immunization consisted of DNP-homologous carrier, but not DNP-heterologous carrier. This provided the first demonstration that the carrier as well as the hapten contributes specifically to the response. Two similar observations were made shortly thereafter by these researchers. They found that strain 2 guinea pigs did not produce antibody in response to poly-L-lysine (PLL) challenge while strain 13 guinea pigs did. Likewise strain 13 "responders" but not strain 2 "nonresponders" made anti-DNP antibodies upon DNP-PLL challenge. However, the nonresponders were shown to be capable of responding to DNP if DNP were complexed to another carrier--

methylated bovine serum albumin. The second case concerned the response of rabbits to the tetrameric isoenzymes of lactic dehydrogenase (LDH) (7). Some rabbits responded well to type I and V isoenzymes while others responded only poorly to type I. However, if the poor responders were immunized with a type I and V hybrid molecule they responded quite well to type I. It appeared that both subunits must be recognized and the inability of the non-responders to develop specific antibody was corrected by providing an immunogenic carrier.

Hindsight now enables us to see that in antibody response a strong case for cooperation between two cell types of different specificity should have been evident. However, it was not until the experiments of Mitchison (8) that this was recognized. He transferred to irradiated recipient mice spleen cells from syngeneic donors previously immunized with 4-hydroxy-5-iodo-3-nitrophenylacetyl-ovalbumin (NIP-OVA). A secondary anti-NIP response was obtained upon NIP-OVA challenge but not NIP-BSA challenge as expected. However, a response to NIP-BSA challenge was obtained when the NIP-OVA primed donor cells were supplemented by BSA primed spleen cells, indicating that the lack of carrier specific "helper" cells had prevented the response initially.

## Relationship of the Carrier Effect and T - B Cooperation

A connection between the carrier effect and thymus-marrow cooperation in SRBC response was discovered when Raff, in a system similar to Mitchison's, established by the use of anti-Thy 1 antiserum that carrier specificity was provided by T cells (9).

Additional experiments established that the carrier recognition function was not transferred by carrier specific antibody (10,11). *In vitro* studies employing carrier specific cells made several further contributions to an analysis of cell cooperation. Dutton *et al.* (12) obtained enhanced primary anti-trinitrophenyl (TNP) antibody responses in DNP-SRBC challenged cultures which contained SRBC carrier primed cells. In addition to verifying that carrier function is indeed thymus derived by the use of anti-theta antiserum, they showed that this function can be supplied by thymus derived cells obtained from the spleens of irradiated mice repopulated with thymocytes and SRBC.

## Specificity of Carrier Cells

It has been suggested that T cell antigen specificity differs from B cell specificity (13). Evidence favoring this argument is based largely on functional studies in which hapten-carrier conjugates provided the antigenic stimulation. As described above, sensitization of animals to a carrier leads to an enhanced primary or secondary anti-hapten response, provided the animals are challenged with the hapten-homologous conjugate. To examine specificity in helper cells a number of studies have utilized the hapten portion of the conjugate as the carrier. Instead of measuring cell response to a large number of determinants, this technique allows an assessment of the limited number of T cell clones responding to a single determinant. Thus, one hapten serving as the carrier is conjugated separately to two heterologous substances; the first to make the

hapten immunogenic in the carrier priming stage and the second to provide the antigen against which response is measured.

A number of studies have used this approach and the results have been equivocal. A study by Paul *et al.* (14), using guinea pigs immunized with DNP-BGG in complete Freund's adjuvant, found no anti-OVA enhancement upon DNP-OVA challenge. Mitchison (15) used a transfer system in mice in which spleen or lymph node donor cells, immunized by DNP skin painting or injection of a DNP-heterologous conjugate, produced an anti-BSA serum titer slightly higher than that of controls which received no helper cells. Unfortunately, a control group of recipients receiving unprimed donor cells was not included in this protocol. Furthermore, antilymphocyte serum (ALS) treatment of recipients as well as a huge dose of conjugate were necessary to produce the effect. This experiment then did not necessarily reflect response to a normal challenge dose in mice bearing a normal population of T cells.

Iverson (16) has reported a similar situation of enhancement in which mice previously sensitized to DNP by skin painting were injected with DNP coupled to immunoglobulin (Ig) extracted from 5563 plasmacytoma cells. After three weeks the mice challenged with DNP-5563 Ig, but not 5563 Ig alone, showed serum antibody directed against 5563 Ig. As before, the evidence for hapten specific helper cells is equivocal because no control was employed to exclude the possibility that 5563 Ig was altered to a more immunogenic form of antigen by DNP attachment and hence able to induce antibody without the aid of DNP specific helper cells. In this study as well as Mitchison's, it was not determined whether antibody directed against

the carrier was able to supply an equal amount of help. This point is important because of an observation by Janeway (17) in which the passive transfer of anti-DNP antibody enabled Balb/C mice to mount an anti-idiotype antibody response against an isologous myeloma protein (LPC-1) when challenged with DNP-LAC-1.

Rubin and Wigzell (18), in a system resembling that used by Mitchison (15), excluded the possibility of carrier specific antibody in the helper cell population by elution through an anti-mouse Ig coated column prior to cell transfer. Column eluted cells and a DNP-BSA challenge were administered to irradiated recipients which had been repopulated with DNP primed T cells. Marginal enhancement of anti-BSA response by primed cells was found using as a basis for comparison the effect of unprimed cells.

While T cell carrier specificity for complex antigens is accepted without question, the foregoing results seem to justify doubt that exists as to whether or not hapten specific carrier cells can significantly enhance responses. The most probable explanation for an inability of hapten priming to effect hapten specific help is an inefficient or defective activation of precursor helper cells. Support for this explanation can be found in recent studies which show that while T lymphocytes are capable of directly binding haptens (19,20), the affinity of cells for the inducing hapten does not change with multiple antigen exposure as is typical of B cells (20).

## T Cell Regulatory Influence: Selective Pressure on B Cells

While the role of the macrophage in the collaborative effort of antibody response cannot be ignored, it is the T cell that most directly participates in this reaction. Although T cell activity is not an absolute requirement for response, the absence of such activity decidedly affects antibody synthesis both quantitatively and qualitatively. This is readily apparent in studies employing mice having either experimentally or genetically induced T cell deficiencies. Based on the most recent evidence, this influence is regulatory in nature and can be viewed as affecting B cells in the following ways.

### Class of Antibody

The influence of T cells on antibody production is perhaps best illustrated by deletion of such influence. One suitable model of nature is the genetically athymic (nude) mouse. While IgM levels of such animals were found to be comparable to that of normal littermates, IgA and $IgG_1$ levels were 10% or less of normal value and $IgG_{2a}$ and $IgG_{2b}$ levels were 20% or less of normal (21,22). Transplantation with either a thymus graft or a thymus cell suspension allowed these mice to produce normal levels of IgA, IgG, and $IgG_{2a}$ within a short time (23). At least for IgA the deficiency was found to be the result of a low synthetic rate rather than a high catabolic rate (21). These data led to the conclusion that the synthesis of these immunoglobulins is T dependent.

Antibody production in response to antigen challenge parallels this situation quite closely. In comparison to normal mice, nudes

produced less IgM antibody and virtually no IgG antibody upon SRBC immunization (23,24). The transplantation of thymocytes alleviated this deficiency and allowed a normal IgG and IgM response to develop (24).

The use of T independent and T dependent antigens in experimentally T cell depleted mice has provided additional evidence that these cells regulate antibody synthesis. Hymphrey *et al.* (25) found that neonatally thymectomized mice developed normal antibody responses to pneumococcal polysaccharide while Armstrong *et al.* (26) similarly showed that bone marrow reconstituted, lethally irradiated mice produced normal antibody responses to purified polymerized flagellin. T independence has since been shown for a number of antigens by various groups (for review see 27). Further analysis of these so-called T independent antigens revealed that they have in common a structure consisting largely of identical repeating units which probably favor a direct positive immunogenic stimulation on contact with B cells. By way of contrast it was hypothesized that T dependent antigens, lacking this structure, would present only ineffectual or tolerogenic signals to B cells in the absence of T cells (27).

Examination of responses to both types of antigen in T depleted mice verified this prediction. Reactions to T dependent antigens were quite poor while those against T independent antigens, as in nude mice, were confined almost exclusively to IgM antibody with secondary responses completely absent (27). Additionally, the absence of T cells considerably narrowed the immunogenic dose range of T independent antigens (27,28). In conjunction with this and

earlier findings the observation that hapten-specific tolerance can be produced by hapten conjugated to nonimmunogenic carriers (28) suggests that in the absence of T regulation direct interaction of antigen with B cells leads to unresponsiveness where it would normally lead to response.

Further evidence demonstrating the T cell influence on class of antibody produced by B cells comes from experiments which showed that both IgG and IgE are T dependent and that different carrier molecules of hapten-carrier conjugates as well as different modes of sensitization favor the development of one or both types of response (29,30). In mice, for example, BGG was a poor carrier for IgE while keyhole limpet hemocyanin and ascaris extracts were good inducers of both IgG and IgE.

In a T dependent antibody response a characteristic "shift" from early IgM production to a more slowly developing IgG production normally occurs. At the time of a secondary response the level of IgG is several times greater than IgM. The T dependence of the development of the IgG component was shown in the experiments of Miller *et al.* (31) in which a shift in the class of anti-3,5-dinitro-4-hydroxyphenylacetic acid (NNP) antibodies induced in response to NNP-horse erythrocytes (HRBC) was observed. Mice not presensitized to the HRBC carrier produced primarily IgM antibodies while carrier primed animals developed predominantly IgG antibodies in the same time period.

## Maturation of Response

Another sphere of the T regulatory influence is maturation of the antibody response. While one aspect of this has already been covered in the discussion of IgM and IgG kinetics, a second concerns T cell influence in the change which occurs in antibody affinity over the course of a response. Affinity in nude, T deprived or even normal mice subjected to challenge by a T independent antigen remains constant while a challenge with T dependent antigen (in mice with T cells) results in a gradual selection for higher affinity antibody. This is exemplified in a study by Gershon and Paul (32) using adult thymectomized and lethally irradiated, bone marrow repopulated mice as recipients of various numbers of syngeneic T cells. Following immunization with either DNP-BSA or DNP-KLH, the affinity of anti-DNP antibody was measured. It was found that while $33 \times 10^7$ cells restored to respectable levels both the amount and affinity of anti-DNP antibody in DNP-KLH challenged mice, a threefold increase in cells administered to the DNP-BSA challenged group did not yield an equivalent effect. The explanation that followed was that the difference in T cell numbers required to restore normal response characteristics represents the difference in carrier specific T cells induced by each antigen. The T cell selection operating here may be indirect and influencing the rate of B cell proliferation (33).

## Suppression: A Phase of Regulation

So far this review has considered only enhancement of B cell response by T cells. However, operationally regulation encompasses a whole spectrum of modulatory control. In terms of T cell activity

this is currently referred to as helper and suppressor function. With regard to the latter, a great deal of evidence now attests to the fact that T cells are capable of protecting the host from over-reaction to immune stimulation by limiting responses. Cells with suppressor function may also play an active part in establishing tolerance. The discussion which follows will consider findings most relevant to the evolvement of current concepts of suppression as they pertain to regulation.

One of the earliest indications that suppressor cells serve as a normal component of the immune system came from experiments demonstrating enhancement of response as a result of T cell depletion. The usual effect of treatment with cytopenic doses of antilymphocyte serum (ALS) is the reduction or elimination of T cell participation in antibody response. In contrast, Baum *et al.* (34) found that ALS treated rats were able to produce an antibody response to KLH that was several times greater than that of control rats given normal serum! This same dose of ALS, in another group of rats immunized with SRBC, prevented the development of a primary response entirely. Enhancement of antibody response against a different T independent antigen by T cell depletion was shown by Baker *et al.* (35,36). Injection of several different strains of mice with one dose of ALS at the time of pneumococcal polysaccharide Type III (S III) immunization resulted in a heightened response. The interpretation was that while T cells favor the development of responses to T dependent antigens, they discourage response to partially or wholly T independent antigens.

Tada and his colleagues verified and extended these findings in rats (37). They initially reported that splenectomy and thymectomy of three-month-old rats subsequently immunized with DNP-ascaris extracts (As) greatly enhanced the formation of homocytotropic antibody (HTA) over a long period. In later experiments they showed that the passive transfer of $10^9$ thymocytes or spleen cells from donors that had been hyperimmunized with DNP-As or As alone were very inhibitory to HTA production. In contrast, cells hyperimmunized to DNP-BSA showed no such effect, thereby indicating specificity (38).

McCullagh (39) likewise presented evidence which suggested that a factor with the capacity to suppress normal cells was induced, although his experiments did not incriminate the T cell as the responsible cell. Normal syngeneic thoracic duct cells transferred to rats made tolerant to SRBC by repeated antigen injections did not overcome the tolerance. The fact that the establishment of grafted normal cells in the host occurred was demonstrated by the ability of these cells to respond to SRBC if recovered from the host before day three. This excluded the possibility that tolerance was not broken because the transferred cells were unable to establish themselves in the host. The tolerant host's B cells did produce antibody if allogeneic instead of syngeneic donor cells were transferred. This allogeneic effect, later the object of more intense research, will be further discussed under Mechanisms of Regulation.

Gershon and Kondo (40) investigated T cell suppression by performing the following experiments. Thymectomized and lethally irradiated mice protected by bone marrow were given tolerogenic

amounts of SRBC over a period of four weeks. Four days after the final injection the recipients were administered thymocytes and an SRBC challenge. By assessing hemagglutination titers, it was found that B cells from these animals were not significantly affected by the tolerogenic regimen. Similarly treated groups, which received a small number of thymocytes in addition to the marrow cells, showed a decided impairment of response. The further addition of normal thymocytes failed to restore response, suggesting that T cells were suppressing B cells either directly or through intermediate T cells.

In another series of experiments (41), B cells from unresponsive mice were found to produce anti-SRBC antibody if normal thymocytes were injected and the animals were subsequently stimulated with a second antigen such as horse erythrocytes (HRBC). These experiments were later interpreted to mean that a suppressor product induced by the initial interaction of T cells and the tolerogenic dose of SRBC inhibited the cooperative functions of normal T cells. It was concluded that the second antigen (HRBC) provided a stimulus which blocked the suppression (42). Still other experiments indicated that this suppression was antigen specific. Normal animals confronted with spleen cells from unresponsive donors were unable to produce a normal anti-SRBC response but yet were able to mount a normal response against HRBC (43).

Tada (44) has recently described two soluble factors extracted from the lymphocytes of KLH primed mice (also discussed below). These have enhancing and suppressing effects respectively on antibody production. The suppressive factor was described to be less than 100,000 MW in size, susceptible to ATS as well as anti-H-2 serum

treatment, and partially inhibited by absorption with column bound specific antigen. The factor suppressed anti-DNP response when either animals or cell cultures were challenged with DNP-KLH and its suppressive activity was largely confined to the indirect or IgG phase of the response.

Kapp and her colleagues (45) used strains of mice genetically nonresponsive to random copolymers of certain L-amino acids to investigate suppressor cells. They found that the inability of these strains to produce antibody against particular amino acid copolymers lies within T cells, not B cells, because responder T cells and nonresponder B cells when mixed yield a normal response. Of particular interest was the finding that immunization of non-responder strains resulted in the induction of antigen specific suppressor cells. Such suppressor cells were sensitive to anti-T cell sera and unlike antigen-induced helper cells were radiosensitive. An active factor was isolated from populations of suppressor cells by the sonication and ultracentrifugation technique used by Tada (44). Similarly, the factor activity was absorbed by the inducing antigen and could be inhibited by an alloantiserum directed at the K and I region of the histocompatibility gene complex.

Eardley and Gershon (46) provided evidence that the suppressor cell induction method used by Tada (44) involved a feedback control mechanism. In their study carrier primed spleen cells were transferred to normal mice which were subsequently challenged with hapten-carrier conjugate. This resulted in a suppressed antihapten response. In contrast, however, these same carrier primed cells in irradiated recipients resulted in an enhanced response. These results are

consistent with a T-T interaction whereby in the first instance too much feedback product reaches unprimed T cells from the carrier primed T cells. In the irradiated recipients the feedback product is unable to influence unprimed T cells and hence is not able to shut off the response before it gets started.

## Mechanism of B Cell Regulation by T Cells

### Unresolved T Cell Considerations

Because our knowledge of T cells is still incomplete, several basic questions must be answered before an understanding of regulatory mechanisms can be approached. Two questions of particular interest that have attracted a great deal of research effort are 1) the nature of the T cell receptor and 2) the interrelationship of helper cells, suppressor cells and cells responsible for cell mediated type reactions.

With regard to the first question, immunological specificity unequivocally exists for T cells. This initially became apparent through delayed hypersensitivity studies (5), specific tolerance induction experiments (47), and experiments demonstrating specific activation by antigen or inactivation by highly radioactive antigen (48). It is agreed among researchers that specificity is necessarily conferred by some type of receptor. B cell receptors are immunoglobulin (Ig) in nature (for review see 13), but the nature of the T cell counterpart remains locked in controversy. At the center of confusion are reports implicating as the receptor an immunoglobulin of either conventional or unknown type, an immune response

(Ir) gene product, or a combination of Ir and histocompatibility (H-2) gene products (49,50,51,52).

Such claims are grounded either in studies involving inhibition of function and antigen binding by specific antisera or the isolation of a small amount of immunoglobulin from cell surfaces. In the case of the inhibition studies conflicting results and the inability of other researchers to confirm these findings make most results questionable. These discrepancies could well be accounted for by the use of nonstandardized antisera or techniques used by the different researchers. Offered also are alternative interpretations of the data, the most common being that steric hindrance results from the coincidental but nonfunctional presence of the determinants selected for by the antiserum. Immunoglobulins supposedly isolated from T cell surfaces might be explained by the presence of small numbers of contaminating B cells in the sample preparations.

In addition to the question of specific receptors, a second major question yet unresolved is whether or not T lymphocytes can be separated into functional subpopulations. This problem has been the subject of much research as well as speculation and is reviewed by Greaves *et al.* (13). It can be simply stated that a considerable body of evidence argues for the existence of T cell subpopulations. Although there is wide disparity from study to study and overlapping functions are often not ruled out, these functional categories might be listed as cytotoxicity, cell mediated immunity and helper function. It remains to be shown that these categories represent unique terminal differentiation pathways. It can be argued that these functions are not mutually exclusive and that their respective

mechanisms in these functions are actually quite similar. Cited as evidence for this are recent studies showing that various functions of these cells involve mediation by one or more soluble factors (33,44,50,53).

Antigen Concentration

One explanation for the role of T cells in cooperation with B cells which achieved early popularity was the "antigen focusing" hypothesis (54). This theory assigned the specific helper T cell a totally passive role in recognizing, binding and conveying antigen to sites of B cell congregation at which point the antigens are in the proper form and position for B cell stimulation. This relatively simple theory was appealing because T cells, as the major component of recirculating lymphocytes, were anatomically in the perfect position to assume this task. The explanation was that in T-B interaction T cells recognize certain determinants of a complex antigen other than those recognized by B cells and hence form an antigen "bridge" to the determinant recognized by B cells.

The evidence for an antigen concentration mechanism is based largely on the *in vivo* experiments of Mitchison (15) and the *in vitro* experiments of Kettman and Dutton (55) which show that for anti-hapten response to occur the carrier of the challenge conjugate must be the same one used in priming and must be attached to the hapten. Free carrier injected with the hapten on a heterologous carrier is ineffective. This work is today open to question because of the possibility that antigenic competition developed under the conditions of the experiments and hence inhibition might have occurred in the presence of the free carrier.

A variation of the antigen focusing concept elaborated by Mitchell and Miller (56) postulated the existence of an IgX molecule as the specific T cell receptor. In either the bound or released form such a receptor could serve equally well as the antigen concentration mechanism. This relaxed the requirement for physical proximity of T and B cells in interaction so that the findings of Feldman (57), which demonstrated *in vitro* T cooperation in antibody response across a cell impermeable membrane, could be explained.

If the T cell was indeed merely a vehicle for antigen presentation it was reasoned that the use of antigen coated particles or cells should mimic the action of these lymphocytes. With this rationale a number of studies were carried out. Katz *et al.* (58) substituted anticarrier antibody coated resin particles for carrier primed lymphocytes in the guinea pig and were unable to find a cooperative effect following hapten-carrier challenge. Physiological extrapolation was of course not possible because it was recognized that polyacrylic particles are not lymphocytes. Unanue (59), by substituting KLH bound to live macrophages for lymphocytes, hoped to restore anti-KLH antibody response in thymectomized mice. Because macrophages do localize in lymphoid areas containing B cells, one objection to the use of inert particles was overcome. However, the question remained unresolved because cooperation did not result.

The most elaborate approach employed carrier tolerant lymphocytes as a passive antigen carrier (60). Fowl gamma globulin (FGG) tolerant murine B and T cells were coated with FGG and given to irradiated recipients in addition to B cells. These recipients

responded to a heterologous antigen but not FGG. However, the use of nontolerant FGG coated T cells did result in a response. These results were verified by a second series of experiments in which a hapten-carrier conjugate served as the antigen. In this case neither carrier nor hapten-carrier conjugated B cells allowed a response unless normal T cells were also administered.

These experiments strongly suggested that T cell participation involved more than simply antigen concentration. Subsequent to the completion of these studies, other approaches were employed in the dissection of the T cell mechanism of cooperation and the results of a number of these investigations appear below.

Genetic Considerations

In a broad sense all immune responses are subject to genetic control since they depend ultimately on gene products. Recently, however, experimental evidence has shown this genetic control to reside in lymphocytes, to be very precise, and to be manifested in a variety of ways (reviewed in 61). Investigation in the area of genetic control has progressed rapidly because of the great impetus provided by the discovery of histocompatibility-linked immune response (Ir) genes. These genes, which determine responsiveness to a wide variety of antigens, have now been identified in a number of species (61).

Macromolecular transfer of genetic information between T and B cells as an explanation for T-B cell interaction has been rejected. Mitchison *et al.* (11) demonstrated this by the use of an adoptive transfer system in mice. When cells from carrier primed donors

differing in allotype from hapten primed donors were mixed in irradiated recipients, the antihapten antibody resulting from hapten-carrier challenge was of the hapten-primed donor allotype. This was confirmed by Jacobson *et al.* (62) in a similar approach which employed SRBC as the antigen. With the elimination of genetic transfer, the search for a control mechanism was narrowed considerably. It was soon established that Ir genes operate primarily, if not entirely, at the T cell level (for review see 50). This conclusion is based on the fact that only T dependent antigens are subject to Ir gene control and that these genes control the recognition (carrier) function. This latter point was demonstrated by the failure of individuals lacking a particular Ir gene to mount a response to the Ir restricted antigen. When the antigen was attached to a non-Ir-restricted carrier, a normal response developed (63). The discriminating capability of Ir control, its manifestation in T cells, and the inability of researchers to conclusively isolate an immunoglobulin antigen receptor from T cells, makes it tempting to equate the T cell clonal receptor with the Ir gene product.

However, the situation is considerably complicated by a number of findings. Optimal murine T and B cell interactions to non-Ir-restricted antigens occurred when each cell type shared gene identities in the K or I region of the histocompatibility (H-2) complex (51). In other experiments employing an Ir controlled antigen and responder and nonresponder strains of mice as well as the responder x nonresponder $F_1$ cross, additional facts came to light (51). If the specificity of the antibody response assessed was unrelated to

that governed by the Ir gene, $F_1$ T cells could provide helper function for responder B cells but not for nonresponder B cells. This indicated that either Ir genes were expressed on B cells after all, though nonclonally, or that two separate gene products were involved with one activating the other. Within a short time several laboratories, using antisera prepared in recombinant mice differing only at genes in the I region, reported reactivity of these sera with surface antigens found primarily on B cells and macrophages and only to a small extent on T cells (reviewed in 61). This opened the possibility that these so-called Ia genes coded for a cell interaction product distinct from the product of Ir structural genes (51).

Lending support to such a hypothesis are the findings concerning an allogeneic effect factor (AEF) described by Katz *et al.* (64) and the soluble factors discussed below. AEF appeared to be most effective if the producing strain of mouse shared with the reacting strain an identity in the K and/or I region of the H-2 complex. Because its activity can be absorbed by appropriate anti-Ia or anti-H-2 sera, this product may in fact be identical to the cell interaction product. Additionally the enhancing factor of Taussig (53) and the suppressing factor of Tada (44), which could also correspond to an interaction product, are inhibitable by anti-H-2 sera.

## Soluble T Cell Products

A growing list of lymphocyte produced substances includes cell receptors and H-2 products as well as a number of specialized factors

which may or may not eventually be traced to H-2 origins. The possibility that all T cell functions, cellular as well as antibody response related, are mediated in large part by soluble factors is today quite favorably received. The elaboration of such factors may indeed be the primary response of this cell just as antibody production is the primary means of B cell expression. Until a cell product common to several functions or a basis for linking the known products to the variety of T cell functions is recognized, such a discussion will necessarily appear fragmentary. For purposes of simplification, T cell products in this discussion will be classified according to specific or nonspecific effects on B cells.

In the nonspecific group, perhaps the most widely studied factor is elicited by an interaction of T cells with histoincompatible lymphocytes. The general phenomenon, termed the allogeneic effect, was first considered in detail by Katz *et al.* (65) when it was found that injection of strain 2 guinea pig lymphocytes into DNP-OVA primed strain 13 guinea pigs caused a significant rise in antibody production. It was subsequently observed that the allogeneic effect could abrogate the need for carrier primed cells since a DNP-BGG challenge could serve as the antigen in eliciting DNP response. While the adoptive transfer of BGG primed syngeneic cells was necessary to help a response under normal circumstances, under allogeneic conditions the transfer of nonprimed cells was adequate. The peak of response appeared six days after allogeneic transplantation. It was found that even a nonimmunogenic carrier such as the copolymer of D-glutamic acid and D-lysine could be used in eliciting this reaction (65).

With respect to regulatory function, the findings allowed two possibilities: 1) the graft vs. host reaction results in a proliferative response of host T cells, making more helper cells available at the time of challenge or, alternatively, 2) the reaction exerts a facilitative effect on B precursors or the antibody forming cells themselves, thus altering their subsequent response to antigen. The latter alternative is favored because it was observed that antibody responses against antigens for which no T cells exist can be elicited (66) and B cell memory was enhanced after allogeneic stimulation and secondary challenge (65).

Dutton *et al.* (67) have demonstrated enhanced anti-SRBC response of normal and T cell-depleted spleen cultures by the addition of a cell-free supernatant from 24-hour mixtures of allogeneic spleen cells. Further study by Amerding and Katz (64) has led to the partial purification of this allogeneic effect factor (AEF) from short term allogeneic supernatants. They reported that a highly active protein moiety of the extract, in the 30,000-40,000 MW range, was able to act directly on B cells in the presence of antigen. Although not antigen specific, the factor was apparently strain specific since for effect it required within the H-2 region a shared identity with the responding cell.

Schimpl and Wecker (68) described a thymus replacing factor (TRF) which was obtained by the stimulation of spleen or lymph node cultures with either alloantigens or concanavalin A. TRF appeared to be remarkably similar to AEF. It was found to exert an influence relatively late in antibody response at 24-60 hours. Unlike AEF,

this factor operated across an H-2 barrier, but this was probably due to the different concentrations used in the two studies.

In addition to allogeneic stimulation, a number of substances such as phytohemagglutinin, pokeweed mitogen, concanavalin A, and small doses of antilymphocyte sera are known to enhance antibody response both *in vivo* and *in vitro* (13). The action of these agents is a nonclonally restricted T cell stimulation which leads to changes reminiscent of antigen stimulation. Increased antibody production may then be a consequence of the increased numbers of available T cells and/or the elicitation of soluble T cell factors such as AEF.

A number of researchers have described various antigen specific T cell factors which regulate B cell antibody responses. Feldman (49,57), using culture chambers with two compartments separated by a cell impermeable membrane, reported effective collaboration across the membrane by T and B cells. KLH primed T cells, but not heterologously primed or nonprimed T cells, collaborated to produce an effective response against DNP-KLH challenge in the B cell chamber. It was subsequently observed that the factor acted indirectly on B cells via macrophages. Because the factor was sensitive to anti-kappa and anti-mu chain antisera, it was concluded that cooperation was mediated via T cell immunoglobulin-antigen complexes. From this it was hypothesized that the essential feature of the mechanism is the proper presentation to the B cell receptors of a "lattice" of appropriately spaced antigenic determinants.

Tada and colleagues (44) have obtained specific *in vitro* anti-DNP enhancing and suppressing factors upon the mechanical disruption

of ascaris extract-DNP primed rat or mouse thymus and spleen lymphocytes. The more completely characterized suppressive component with a molecular weight of less than 100,000 was found to be sensitive to antithymocyte as well as anti-H-2 sera. Although it resembled the nonspecific allogeneic factor in molecular weight, it was not a T cell replacing factor and was active only in the presence of T cells. While *in vitro* work on the enhancing factor of 100,000-200,000 MW was reported to be progressing more slowly, from *in vivo* studies it appeared that specificity was of the same degree as the suppressive factor. Thus it is possible that the two molecules share some antigen recognition moiety.

Taussig and Munro (53) have described in the mouse system a T replacing factor which they feel is H-2 linked and is the soluble expression of the T cell receptor. Factor containing supernatant was prepared by educating thymus cells in irradiated recipients with the synthetic polypeptide poly(tyr,glu)-poly DL ala- - poly lys [(T,G)-A- -L]. After six days the cells were recovered from the spleens of these donors and cultured with the priming antigen for 6-8 hours. This factor, when given to B cell reconstituted recipients challenged with (T,G)-A- -L, resulted in a response. There was no response if the animals were unprimed or heterologously primed. Anti-Thy 1 treatment of cells cultured with the antigen prevented elicitation of the factor and the factor itself was inhibited by antisera specific for the I region of the H-2 complex. Again this substance bears some resemblance to the enhancing factors described previously despite the fact that it operates across H-2 barriers.

In summary, it is clear that the mechanism by which T cells regulate antibody response is far from being resolved. Simple theories such as antigen concentration which rely on passive T cell participation are no longer adequate in explaining the data. The evidence for genetic determination of responsiveness through the elicitation of lymphocyte factors now seems firmly rooted. At least superficially, a number of these products are similar and others, particularly those combining specificity and function, might be accounted for by the association of an antigen receptor and a second factor. It is also quite possible that there exists an identity or at least some degree of structural homology between these products and those mediating cellular responses.

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# REGULATION OF ANTIBODY RESPONSE BY DNP CARRIER-PRIMED CELLS

## I. DNBS INDUCED SUPPRESSION

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(ABSTRACT)

Dinitrochlorobenzene (DNCB) produces antigen specific contact sensitivity in mice. It was previously shown that pretreatment of the animals with dinitrobenzene sulfonate (DNBS) renders them tolerant to subsequent DNCB sensitization. In the present study, DNCB skin sensitization did not induce spleen cells capable of adoptively transferring helper activity to mice challenged with DNP-SRBC upon assay for anti-SRBC response. Cells from mice treated with 15 mg of DNBS did, however, exhibit a suppressive effect in this system since direct and indirect anti-SRBC PFC responses were consistently lower than those of control groups receiving normal cells. Such suppression was also evident in a secondary response. DNBS induced suppression appears to be antigen specific since suppression of the response did not occur when SRBC were substituted for DNP-SRBC as the challenge antigen. The suppression routinely achieved by the transfer of 1-2 x $10^7$ cells did not improve by transfer of more ($10^8$) cells, but did decline with the transfer of fewer ($10^6$) cells. At least partial abrogation of suppression in both primary and secondary responses was achieved by treatment of donor cells with rabbit anti-BA-Thy 1 serum.

## INTRODUCTION

Certain populations of thymus derived (T) lymphocytes have the capacity to regulate some aspects of terminal differentiation by bone marrow derived (B) lymphocytes (1). This role is exemplified by the 'carrier effect' first described by Ovary and Benacerraf (2). Rabbits primed with dinitrophenylated bovine gamma globulin (DNP-BGG)

produced a secondary response to DNP only if the secondary challenge was by DNP on the homologous carrier. Since observed in other experimental systems, the effect is currently best explained by cell cooperation (3). In the most basic terms, T cell immunity to the carrier determinant of a multideterminant antigen enhances the B cell response against a second determinant.

Ordinarily the protein moiety of a hapten-protein conjugate is used as the carrier determinant in this type of a system. The multiclonal T cell response evoked by the complex antigen then provides the T component of cooperation. However, the use of a hapten determinant as the carrier moiety, by restricting the clonal response, could considerably simplify an examination of the T cell regulatory role in this system.

DNP has often been used in immunological studies because it is conjugated readily to other molecules and is available in a number of forms (4). The selective use of a certain chemical form or immunization procedure can then be exploited in producing a certain type of response. For example, DNP contact sensitization of mice by dinitrobenzene compounds is known to evoke primarily a T cell response, as shown in histological studies (5,6), yet does not lead readily to antibody production (6). By the intraperitoneal route, DNP-protein conjugates have been shown to result in DNP binding T cells (7,8).

In an earlier study we used a radiolabeling procedure to partially quantitate T lymphocyte participation in a DNCB induced contact sensitivity response. Treatment of test animals with a large amount of dinitrobenzene sulfonate (DNBS) prior to sensitization eliminated

this response (9), as had been previously reported by other researchers who used tissue swelling as an indicator (5,10,11). Various attempts to show DNP-specific carrier T cell help in boosting antibody response by DNP skin sensitization or DNP-protein conjugate immunization have been marginally successful (12-16), but as pointed out, sometimes incomplete in controls (17). These studies often required a particularly sensitive assay to detect an increase in antibody titer or did not exclude the possibility of conjugate specificity. In the work presented below, it was expected that such helper cells would recognize the DNP determinant of a DNP-SRBC challenge and help the host B cells produce a greater anti-SRBC PFC response (over and above that observed with unprimed cells). By similar logic, DNBS primed cells which abrogate or suppress DNP contact sensitivity (18), when used in such an adoptive transfer system, were expected to eliminate such help or even suppress an antibody response to SRBC. Experimental results presented are inconsistent with the first of these expectations, but provide evidence for a specific suppressive effect.

## MATERIALS AND METHODS

Mice. (C57Bl/10 x C3H/He) $F_1$, 8 to 16 week old female mice were obtained from Cumberland View Farms (Clinton, TN) or Health Research, Inc. (West Seneca, NY) for use as donors and recipients in all experiments. CBA mice from Jackson Labs (Bar Harbor, ME) were the source of antigen in the preparation of anti-brain associated Thy-1 antiserum (Anti-BA-Thy 1).

Antigens. SRBC were obtained from a single sheep housed at the Williams Sheep Farm, Mason, MI. Cells were stored in Alsever's solution and when used without modification washed three times in phosphate buffered saline (PBS) and injected iv at $5 \times 10^8$ cells/mouse. Dinitrobenzene sulfonate (DNBS) (Eastman Kodak, Rochester, NY) for direct injection as well as for DNP coupling to SRBC was recrystallized twice in ethanol to remove impurities (19). For injection, DNBS was resuspended in PBS and injected iv in a volume of 0.5 ml. Except for minor modifications, the procedure used for coupling DNP to SRBC is described by Rittenberg and Pratt (20). SRBC were washed three times in modified barbital buffer (MBB) and 1 ml packed cells was then added dropwise with stirring to 100 mg DNBS dissolved in 7 ml cacodylate buffer (pH 6.8). After stirring for 30 min at 25 C the reaction was stopped by washing the cells in 50 ml MBB, followed by two further washes in MBB containing 1 mg/ml glycyl glycine. Cells were then resuspended in PBS, mixed with donor spleen cells, and immediately injected at $5 \times 10^8$ DNP-SRBC/mouse. For preparation of TNP-SRBC, trinitrobenzene sulfonate (TNBS) was substituted for DNBS. Dinitrophenylated bovine gamma globulin (DNP-BGG), prepared as described by Nisonoff (19), was spectrophotometrically determined to have a DNP:BGG ratio of 15.

Sensitization. Mice were primed by applying with dinitrochlorobenzene (DNCB) in olive oil to the clipped abdomen 5 and 3 days prior to harvesting of cells (9,10,11). DNP-BGG sensitization was carried out by intradermal injection with 100 µg initially, and 50 µg thereafter of DNP-BGG in complete Freund's adjuvant (1:1) at

6 months, 5 months and 1 week prior to harvesting of spleen cells. Dinitrobenzene sulfonate (DNBS) primed cells were routinely prepared by intravenous injection of 15 mg DNBS in 0.5 ml saline 7 days prior to use. Donor cells for group C in Table 1 were injected on day -7 and -3 with 1 mg DNBS.

Cell preparation and transfer. Thymus, bone marrow and spleen cells were properly dispersed in Eagle's minimal essential medium (MEM) as previously reported (21). Cells were then washed, counted by means of eosinophil counting chambers, mixed with the DNP-SRBC antigen preparation and injected into a lateral tail vein. Complete details of these procedures are described elsewhere (21).

Preparation of rabbit anti-brain associated Thy 1 (anti-BA-Thy 1) serum. Rabbits were injected intramuscularly with a CBA mouse brain/complete Freund's adjuvant emulsion (22) twice, initially with a 1 month interval and then subsequently 1 week prior to each bleeding. Sera were collected, absorbed with mouse liver and agarose, and then tritiated by measuring cytotoxicity against mouse thymocytes. Serum used in these experiments showed >95% killing of $10^6$ cells at a 1:200 dilution.

Antiserum treatment of cells. To a washed single cell spleen suspension was added 0.5 ml/spleen of anti-BA-Thy 1 serum, 4 ml MEM and sufficient complement to yield a final 1:10 dilution. After 45 min incubation at 4 C the cells were washed twice and finally resuspended in serum free MEM.

Plaque forming cell assay. Spleen cells were assayed by the Jerne hemolytic plaque assay as modified for use with agarose gel, details of which are described by Miller and Cudkowicz (21). Guinea pig complement (Suburban Serum Labs, Silver Springs, MD) was used to develop direct plaques and rabbit anti-mouse γ-globulin (Cappel Labs, Downington, PA) was used (at a dilution inhibitory for 90% of direct PFCs) for indirect plaque development.

## RESULTS

Demonstration of suppressor but not helper activity with DNP primed cells. This study was undertaken in an attempt to determine how carrier cells influence antibody response. Because this type of T cell function cannot be measured directly, the best alternative would be to assess the number of hemolytic plaque-forming cells (PFC) resulting from a T-B cell interaction. We achieved this by adoptive transfer of a cell population (containing DNP-primed carrier cells) to recipients which were simultaneously challenged with DNP derivatized sheep red blood cells (DNP-SRBC) with the rationale that DNP reactive cells, in their capacity to act as carrier cells, would ultimately influence the number of PFCs responding to SRBC. The protocol is summarized in Figure 1.

Primed and unprimed spleen cells, harvested from animals at the time in which they maximally elicit or inhibit contact sensitivity, were injected into normal recipients which were simultaneously challenged with DNP-SRBC. The results of the day 5 assay shown in Table 1 indicate that DNCB priming did not boost the PFC response. In fact, this priming reduced the number of direct PFC/spleen from

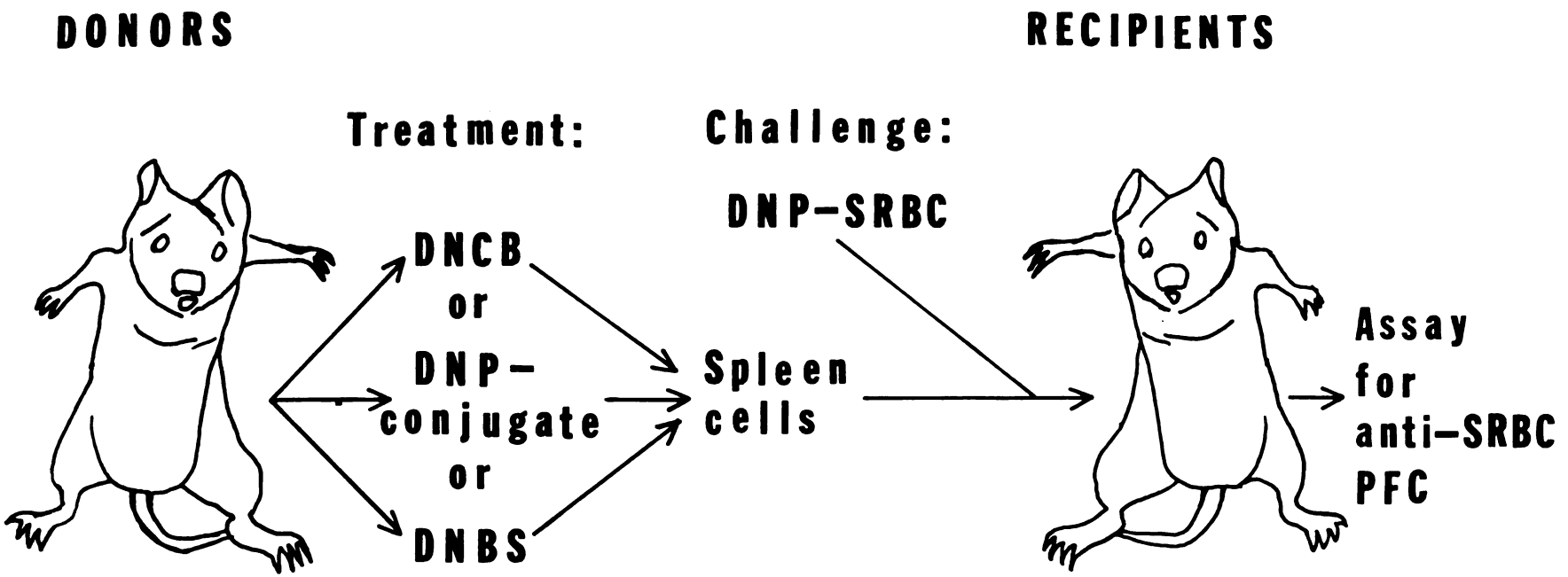


Figure 1. Schematic protocol for determining the influence of DNP-primed donor spleen cells on the anti-SRBC PFC response of recipients challenged with DNP-SRBC. Controls consist of unprimed spleen cells substituted for primed cells. Percent change is determined by the formula:

$$\frac{\text{Norm PFC - Test PFC}}{\text{Norm PFC}} \times 100.$$

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Table 1. Suppression of SRBC response in normal mice by adoptively transferred DNP-primed spleen cells

| Group | Donor Cells ($2 \times 10^7$) | Anti-SRBC PFC/spleen ($\times 10^3$) + SE[a] Direct | Indirect |
|---|---|---|---|
| A | Norm | 242 ± 20 | 273 ± 13 |
| B | DNCB[b] | 175 ± 17 | 255 ± 29 |
| C | DNBS (1 mg)[c] | 241 ± 24 | 212 ± 28 |
| D | DNBS (15 mg)[d] | 227 ± 32 | 205 ± 25 |

[a] p values for significant differences between day 5 responses as determined by 2 tailed Student t test: direct AB<0.05; indirect AC<0.05, AD<0.05. Groups A-C included 10 animals and group D 8 animals.

[b] DNCB donors were treated with 1.5% DNCB in olive oil 5 and 3 days before cell transfer.

[c] Injected iv with 1 mg DNBS 7 and 5 days before cell transfer.

[d] Injected iv with 15 mg DNBS 7 days before cell transfer.

a mean value of 242 to 175 ($\times 10^3$). The transfer of cells from mice treated with 15 mg or 1 mg of DNBS resulted mainly in the depression of the indirect (7s) response.

In two earlier studies reporting hapten induced carrier help it was necessary to partially deplete the host of endogenous T cells by antiserum treatment (12,14). To reduce the possibility that recipient T cells were somehow blocking expression of the transferred cells in the current study, normal recipients were treated with rabbit anti-BA-Thy 1 serum prior to cell transfer. They were then administered $2 \times 10^7$ donor cells and the DNP-SRBC challenge as before. As seen in Experiment I of Table 2, both direct and indirect responses of

Table 2. Suppression but lack of helper activity in anti-BA-Thy 1 treated hosts receiving primed spleen cells

| Experiment and Group | Donor Cells | Anti-SRBC PFC/spleen ($x10^3$) + SE[a] Direct | Indirect |
|---|---|---|---|
| I[b] A | Norm | 215 ± 22 | 195 ± 8 |
| B | DNCB[c] | 140 ± 12 | 147 ± 23 |
| C | DNBS | 119 ± 12 | 128 ± 12 |
| II[d] D | Norm | 264 ± 21 | 141 ± 15 |
| E | DNP-BGG[e] | 226 ± 24 | 136 ± 17 |
| F | DNBS | 165 ± 13 | 136 ± 9 |

[a] p values for significant differences as determined by 2 tailed Student t test: direct AB<0.05, AD<0.01, DF<0.05; indirect AB<0.05, AC<0.01.

[b] Recipients injected with 0.1 ml rabbit anti-BA-Thy 1 serum 1 and 3 days prior to injection of donor cells and DNP-SRBC. Each group, consisting of 6 animals each, was given 2 x $10^7$ donor cells and assayed on day 5.

[c] Donors primed with 1.5% DNCB in olive oil 5 and 3 days before cell transfer.

[d] Recipients injected with 0.1 ml rabbit anti-BA-Thy 1 serum 1 and 3 days prior to injection of donor cells and DNP-SRBC. Each group received 1.5 x $10^7$ cells and was assayed on day 5.

[e] Donors injected with DNP-BGG/complete Freund's adjuvant 6 months, 3 months, and 1 week prior to use of cells.

groups B and C, which received DNCB and DNBS primed cells respectively, were lower than responses of group A, which received normal cells. The suppression by DNBS primed cells was more marked than before and exceeded that produced by DNCB primed cells.

In order to remove the possibility that the mode of priming (skin painting) was not capable of providing adequate stimulation for helper activity, this experiment was repeated using cells primed by an alternate method (Table 2, experiment II). When DNCB primed cells were replaced by DNP-BGG primed cells, the response (226 x $10^3$ PFC/spleen) was not altered with respect to the control (264 x $10^3$ PFC/spleen). However, DNBS primed cells did significantly suppress the direct response down to 165 x $10^3$ PFC/spleen.

It became evident that DNCB and DNP-BGG, as well as DNP-burro erythrocyte priming (results not presented), in this system did not produce helper cells capable of enhancing anti-SRBC PFC numbers upon adoptive transfer and challenge with DNP-SRBC. Suppression, on the other hand, was achieved most efficiently when donor cells were exposed to DNBS *in vivo*. The following series of experiments were then aimed at examining the properties of DNBS induced suppression.

Specificity of suppression. To determine whether this effect was specific, the DNP determinant was eliminated from the challenge antigen by substituting SRBC for DNP-SRBC. If suppression could occur through the recognition of DNP by a carrier cell, SRBC challenge should not elicit suppression. Results shown in Table 3 support this presumption. In two separate experiments the mean response of 10 mice receiving DNBS primed donor cells was compared with that of 10 mice receiving normal cells. The results of these experiments were identical and, when combined, show a level of inhibition of 7.0 and -3.2% for direct and indirect responses, respectively. These data, when compared with the composite mean

Table 3. Specificity of DNBS-spleen cell induced suppression

| Donor Cells | Challenge Antigen | % suppression[a] (Anti-SRBC PFC/spleen) Direct | Indirect | Number of Animals |
|---|---|---|---|---|
| Norm | SRBC | 7.0[b] | -3.2 | 20 |
| DNBS | SRBC | | | 20 |
| Norm | DNP-SRBC | 24.0[c] | 33.4 | 60 |
| DNBS | DNP-SRBC | | | 60 |

[a] % suppression = $\frac{\text{Norm PFC - DNBS PFC}}{\text{Norm PFC}} \times 100.$

[b] Represents the complete result of 2 experiments comparing the effect of normal to DNBS primed cells. Differences in either individual experiment were not statistically significant with $p>0.05$. Recipients received either 1.5 or 2 x $10^7$ donor cells.

[c] Represents the composite mean of 7 experiments in which responses of DNP-SRBC challenged test groups were compared with control groups. The majority of such differences were statistically significant, as is seen in other tables showing results of individual experiments. Recipients received 1, 1.5 or 2 x $10^7$ donor cells.

suppression achieved in 7 experiments in which the challenge was DNP-SRBC, suggest that the effect is specific.

Lack of discrimination between DNP and TNP. Immunization with cross reacting haptens provides a means of limiting clonal selection and hence the heterogeneity of an antibody response (23). For the DNP system this involves primary immunization with a DNP-conjugate and secondarily with TNP conjugated to the same carrier (24,25,26). One study employing this technique had earlier reported that a significant proportion of the different clonal anti-DNP antibodies cross

react (26). Cross reactivity was tested in the current study in an effort to determine whether the T cells involved in suppression, through their receptors, were able to discriminate between DNP and TNP determinants. Table 4 shows the results of such an experiment. Two groups of control mice were given DNBS primed cells. Of these one control and one test group were challenged with DNP-SRBC (A and B) while the other control and test group received TNP-SRBC (C and D). Apparent suppression occurred in the direct and indirect responses of both groups receiving DNBS primed cells, but statistical analysis revealed only the direct response of the TNP challenged group and the indirect response of the DNP challenged group to be significant. Because both the direct and indirect anti-SRBC response are T dependent and significant suppression occurred with both types of challenge, this suggests that T cell receptors, like B cell receptors, are cross reactive for related determinants.

T dependence of suppression in primary and secondary responses. Suppression of responses to T dependent antigens in a majority of systems studied to date has been attributed to T cell influence (1). Restoration of response to control level upon elimination of Thy-1 positive cells would likewise indicate a T cell involvement in the present study. As seen in experiment I of Table 5, such treatment was effective in restoring to control level the indirect component of the response, indicating at least partial T cell dependence of the effect.

Secondary responses are characteristically more T cell dependent than primary responses and exhibit a greatly increased indirect

Table 4. DNBS suppression of anti-SRBC PFC response upon DNP- or TNP-SRBC challenge

| Group[b] | Donor Cells | Challenge Antigen | Anti-SRBC PFC/spleen ($x10^3$) + SE[a] Direct | Indirect |
|---|---|---|---|---|
| A | Norm | DNP-SRBC | 286 ± 42 | 252 ± 26 |
| B | DNBS | DNP-SRBC | 230 ± 17 | 116 ± 9 |
| C | Norm | TNP-SRBC | 240 ± 8 | 191 ± 25 |
| D | DNBS | TNP-SRBC | 158 ± 7 | 175 ± 35 |

[a] p values for significant differences as determined by 1 tailed Student t test: direct CD<0.001; indirect AB<0.005.

[b] Recipients were injected with 1.5 x $10^7$ donor cells and the challenge simultaneously and assayed 5 days later. Groups consisted of 7 animals each.

component. Consequently it is expected that such a response would be at least equally susceptible to DNBS suppression if indeed T dependent. To determine whether or not this was the case, experiment II in Table 5 was performed. Normal mice which had been primed with DNP-SRBC 10 days earlier were given either DNBS primed or normal spleen cells in addition to a second DNP-SRBC challenge. Statistically significant suppression of PFCs occurred in both the direct (NORM:483 x $10^3$; DNBS:336 x $10^3$) and indirect (NORM:798 x $10^3$; DNBS:525 x $10^3$) response at a level comparable to that found in primary responses. The fact that the animals had previously encountered DNP in the primary DNP-SRBC immunization did not seem to alter the level of suppression. Anti-BA-Thy 1 treatment of donor cells prior to transfer did largely restore indirect PFCs to control level and unexplainedly caused direct PFCs to exceed the control level.

Table 5. Loss of primary and secondary spleen cell suppression by anti-BA-Thy 1 treatment of transferred cells

| Experiment and Group | Donor Cells | Anti-SRBC PFC/spleen ($x10^3$) + SE[a] Direct | Indirect |
|---|---|---|---|
| I A[b] | Norm | 180 ± 24 | 57 ± 6 |
| B | DNBS | 138 ± 11 | 20 ± 3 |
| C | DNBS (anti-BA-Thy 1)[c] | 144 ± 14 | 52 ± 9 |
| II D[d] | Norm | 483 ± 34 | 798 ± 45 |
| E | DNBS | 336 ± 47 | 525 ± 43 |
| F | DNBS (anti-BA-Thy 1) | 654 ± 49 | 705 ± 29 |

[a]p values for significant differences of day 5 responses as determined by 1 tailed Student t test: direct AB $0.1>p>0.05$, DE<0.025, EF<0.01; indirect AC<0.01, EF<0.005; indirect AC<0.01, DE<0.005.

[b]Groups A and B consisted of 10 animals each and group C, 8 animals. All three groups were given 2 x $10^7$ donor cells.

[c]Cells treated 30 min with C' and 0.5 ml antiserum/spleen.

[d]Groups D, E and F were injected with DNP-SRBC 2 weeks prior to injection of secondary DNP-SRBC challenge and $10^7$ donor cells. Groups consisted of 10 animals each.

The effect of varying numbers of suppressor cells on PFC response. The preceding experiments involved the transfer of 1-2 x $10^7$ spleen cells and demonstrated a characteristic level of suppression. To determine whether the level of suppression would vary with the number of cells transferred, the following experiment was performed. Each of three groups of normal recipients was injected with one of three different cell concentrations of control and DNBS

primed donor cells. The degree of suppression achieved in each case is shown in Table 6 and was derived by the formula:

$$\% \text{ suppression} = \frac{\text{Norm PFC} - \text{DNBS PFC}}{\text{Norm PFC}} \times 100$$

Table 6. Suppression achieved with the transfer of varying numbers of donor cells

| Group | Treatment | Number | % Suppression (Anti-SRBC PFC/spleen)[b] Direct | Indirect |
|---|---|---|---|---|
| A | Norm | $10^6$ | -32.8 p>0.2 | 13.2 p>0.5 |
| B | DNBS | $10^6$ | | |
| C | Norm | 1-2 x $10^7$ | 24.0[c] | 33.4 |
| D | DNBS | 1-2 x $10^7$ | | |
| E | Norm | $10^8$ | 28.5 p<0.02 | 20.8 p<0.05 |
| F | DNBS | $10^8$ | | |

[a]Normal mice were given donor cells and a DNP-SRBC challenge simultaneously and assayed on day 5. A 1 tailed Student t test was used to determine p values.

[b]$\% \text{ Suppression} = \frac{\text{Norm PFC} - \text{DNBS PFC}}{\text{Norm PFC}} \times 100.$

[c]Represents the composite mean of 7 experiments reported in this paper. Recipients received 1, 1.5 or 2 x $10^7$ donor cells. Statistical evaluation of each individual experiment included is reported in the appropriate table and the majority were significant ($p<0.05$).

To make the data as representative as possible, the percent suppression for all experiments involving the transfer of 1-2 x $10^7$ cells in the primary response was averaged. The value for groups C and D are then a composite mean derived by comparing responses of

50 mice receiving normal cells to that of 50 mice receiving DNBS treated cells.

Evaluation of responses obtained from control and test groups showed that when $10^8$ cells were transferred direct and indirect inhibition levels were 28.5 and 20.8%, respectively. This level of suppression does not show improvement over that produced by the transfer of 1-2 x $10^7$ cells (24.0 and 33.4%). On the other hand, suppression was low or nonexistent and differences not statistically significant upon evaluation of the groups receiving $10^6$ cells.

## DISCUSSION

The major findings of this study can be summarized as follows. 1) Neither DNCB nor DNP-BGG priming of normal mice resulted in an increased PFC response to SRBC when spleen cells were transferred to normal recipients that received a DNP-SRBC challenge. Prior anti-BA-Thy 1 treatment of recipients, described as essential for augmentation of anti-BGG response in a similar system (12,14), failed to improve the response. Instead of enhancement these preliminary experiments marked a general decline in PFC numbers, indicating these methods of hapten priming did not induce helper cells. 2) DNBS primed cells, already demonstrated to specifically abrogate and even suppress contact sensitivity response to dinitrobenzene compounds (5,9,10), also partially suppressed PFC response to SRBC when transferred to normal recipients in the system described above. This suppression, in both the direct and indirect assays and for primary and secondary responses, appeared more efficient than the inhibition achieved by DNCB or DNP-BGG priming. 3) The suppression

resulting from DNBS cell transfer was DNP specific because animals which received DNBS primed cells and SRBC as antigen (instead of DNP-SRBC) showed little or no anti-SRBC PFC suppression. However, DNBS primed cells did suppress the response of animals challenged with TNP-SRBC. Taken together these experiments suggest that T cells involved in the regulation of B cell response are capable of some degree of hapten recognition, as are the T cell mediators of cellular responses and like antibody receptors the T cell receptor cross reacts with structurally similar determinants. 4) Adoptive transfer of $10^6$ DNBS primed cells provided no significant suppression in this system while a level of 1-2 x $10^7$ or $10^8$ cells was able to achieve suppression. 5) DNBS induced suppression was largely eliminated by *in vitro* treatment of donor DNBS cells with anti-Thy 1 serum prior to transfer, suggesting that the effect is T dependent.

These experimental results portray a type of suppression that is reproducible as well as relatively consistent. Mean PFC inhibition achieved over the course of seven experiments was determined to be 24.0 and 33.4% for direct and indirect responses, respectively. These figures were derived by comparing the responses of those control and suppressed mice which had received a range of 1-2 x $10^7$ donor spleen cells. In considering the degree of suppression, one must take into account the fact that erythrocytes bear a large number of strong surface antigenic determinants. Consequently, DNP recognizing regulator (suppressor) cells must compete with a sizable number of other regulator cells recognizing natural determinants and, as a result, the former are not expected to exert total control over the response. Of direct bearing on this point is a preliminary experiment

(unpublished results) in which it was noted that coupling DNP to SRBC under conditions of this study increased the antigenicity of SRBC by approximately 30%. This indicates the added DNP determinant is at least a part of a larger carrier antigen recognized by helper T cells. It might then be logically argued that an equal level of negative regulation by suppressor cells recognizing the same determinant would not be unreasonably small.

The failure to demonstrate helper activity with the transfer of primed spleen cells is not without precedent. Tada and Takemori (27) as well as Eardley and Gershon (28) reported suppression in normal recipients receiving carrier primed spleen cells which were expected to augment antihapten antibody response. This suppression was presumably due to recruitment of suppressor cells of host origin by the antigen-induced donor cells. In the current study partial depletion of host T cells by anti-BA-Thy 1 treatment did not favor the expression of helper activity, nor did it appreciably hinder the activity of the donor suppressor cells. This suggests that suppressor cells are induced in the donor and the activity is conveyed by donor cells and is not dependent on the recruitment of host suppressor cells. The loss of this activity upon anti-Thy 1 treatment of donor cells lends further support to this idea and indicates T cell lineage.

The general mechanism by which DNBS induces suppression is still open to speculation. Simple clonal deletion as an explanation is inadequate for either the cellular or humoral phenomenon. While animals treated with DNBS are subsequently not subject to contact sensitivity induction by dinitrobenzene compounds, this

unresponsiveness is actively transferrable to normal animals by adoptive transfer of lymphocytes (29). In the current study normal animals receiving a small number of such cells were actively suppressed in antibody response and this suppression appeared dependent on the presence of T cells. A likely explanation is then direct T cell control over B cell clonal expansion (30).

The fact that DNP suppressor but not helper cells were readily induced might indicate that the nature of the DNP determinant does not allow proper stimulation for the generation of a positive signal (i.e., for the induction of helper cells). Instead, feedback of suppressing signal is initiated as proposed by Eardley and Gershon (28) to explain suppression by cells expected to be augmentive.

In suppressed animals $10^7$ transferred spleen cells, less than 50% of which are T cells, are able to effect a reduction in response of about $5 \times 10^4$ PFC. This suggests that these cells interact with B cells extremely well or alternatively and more plausibly are also capable of acting by diffusable factor(s). Because T cells have here been shown to function with DNP specificity in a humoral response and elsewhere in a cellular reaction (5,9,10), it may also be argued that the existence of two separate antigen recognition mechanisms (T cell receptors), both of which recognize the same hapten, is unlikely (27). A combination of operation via a diffusable factor and antigenic specificity in a particular response suggests an association between a T cell receptor and a regulatory factor. If this is so, then the case for the association or identity of humoral and cellular response factors elaborated by Taussig (31) might also be strengthened.

To more fully understand suppression and to eventually unravel the basic mechanisms of immune regulation, this model as well as other models under study must be scrutinized carefully for fundamental similarities or differences. Toward this end, additional experiments aimed at further characterization of the nature of DNBS induced suppression are being conducted.

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# REGULATION OF ANTIBODY RESPONSE BY DNP CARRIER-PRIMED CELLS

# II. CHARACTERIZATION OF FUNCTIONAL CELL POPULATION INVOLVED IN DNBS INDUCED SUPPRESSION

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(ABSTRACT)

It was previously demonstrated that pretreatment of mice with a single large dose of dinitrobenzene sulfonate (DNBS) renders them unresponsive to subsequent dinitrophenyl (DNP) skin sensitization. We now describe further functional characteristics of DNBS induced spleen cells with regard to antibody response. Adoptive transfer of such cells to normal recipients partially suppressed their responsiveness to sheep erythrocytes (SRBC) following DNP-SRBC challenge. In the present study this effect was found not to be mediated by serum. The suppressor cells were directly induced by DNBS rather than recruited from the recipient population since B cell reconstituted x-irradiated recipients given DNBS educated thymocytes or spleen cells showed a level of response inhibition similar to that seen in normal nonirradiated recipients given DNBS primed spleen cells. The relative level of suppression apparent upon comparison of responses in recipients of normal or DNBS primed cells was preserved when the responses of both groups were boosted by allogeneic enhancement. C3H parental as well as the C57 Bl x C3H $F_1$ cells used in the preceding experiments were receptive to both the suppressive and allogeneic activity but only in the simultaneous presence of both $F_1$ and C3H parental type T cells. Responses of normal or x-irradiated B cell repopulated $F_1$ recipients were allogeneically enhanced but not suppressed. Possible implications of these findings for a regulatory mechanism are discussed.

# INTRODUCTION

T lymphocytes have come to assume a key role in our understanding of the immune control mechanisms. It is now widely accepted that this regulation may take either a positive or negative form. At present this is still an operational concept because there is yet no basis for determining whether or not helper and suppressor cells are embodied in one multifunctional cell type or differentiate as separate entities. Neither do we understand the relationship of T cells involved in cellular and humoral reactions.

Our previous research approached these problems by asking whether antigen recognition by T cells in different functional states was comparable. Using the dinitrophenyl (DNP) system it was found that dinitrochlorobenzene (DNCB) priming, while inducing a cellular reaction (1), did not induce spleen or peritoneal lymphocytes capable of augmenting plaque forming cell (PFC) response against sheep erythrocytes (SRBC) in an adoptive transfer system where the recipients are challenged by DNP-SRBC (2, and unpublished observations). Likewise, DNP-bovine gamma globulin and DNP-burro erythrocyte priming was also ineffectual. These results contrast with several reports of antibody response augmentation to the protein moiety of a DNP-protein challenge when recipient mice are provided DNP primed cells (3,4,5).

Dinitrobenzene sulfonate (DNBS) priming, on the other hand, was found to result in tolerance induction in the cellular reaction and the induction of cells suppressing PFCs in the humoral reaction (2). These findings suggest that T cell antigen recognition is

similar, at least in the suppressive phase, for cellular and humoral reactions. While this system has not established whether or not separate T cell subpopulations perform the various T cell functions, it has provided a vehicle by which further mechanistic questions concerning control may be posed.

We now report that lymphocytes from DNBS primed mice, unlike the suppressor cells of most other systems, are directly induced by antigen and influence B cells. This study further suggests that T cells produce more than one type of signal which affects B cells since under some circumstances proportionate suppression occurs in spite of a nonspecific response-enhancing stimulus.

## MATERIALS AND METHODS

Mice. (C57Bl/10 x C3H/He)$F_1$ ($BC3F_1$) and C3H/He or C3H/St 8 to 16 week old female mice were obtained from Cumberland View Farms (Clinton, TN) or Health Research Inc. (West Seneca, NY) for use as donors and recipients in all experiments. CBA mice from Jackson Labs (Bar Harbor, ME) were the source of antigen in the preparation of anti-brain associated Thy-1 antiserum.

Irradiation. Mice were exposed to 950 rads of $^{60}Co$ γ-radiation from an emitter contained in the Department of Food Science facility at Michigan State University. Mice were rested for 2-5 hr before transplantation.

Antigens. SRBC were obtained from a single sheep housed at the Williams Sheep Farm, Mason, MI. Cells were stored in Alsever's solution until use. Dinitrobenzene sulfonate (DNBS) (Eastman Kodak,

Rochester, NY) for direct injection as well as for DNP coupling to SRBC was recrystallized twice in ethanol (6) to remove impurities. For injection, DNBS was resuspended in phosphate buffered saline (PBS). The basic procedure used for coupling DNP to SRBC is described by Rittenberg and Pratt (7). Briefly, SRBC were washed three times in modified barbital buffer (MBB) and 1 ml packed cells was then added dropwise with stirring to 100 mg DNBS dissolved in 7 ml cacodylate buffer (pH 6.8). After stirring for 30 min at 25° C in the dark, the reaction was stopped by washing the cells in 50 ml MBB, followed by two additional washes in MBB containing 1 mg/ml glycyl glycine. Cells were then resuspended in PBS, mixed with donor spleen cells and immediately injected at a concentration of $5 \times 10^8$ SRBC/mouse.

Cell sensitization, preparation and transfer. DNBS primed spleen cells were prepared by intravenous (iv) injection of 15 mg DNBS in 0.5 ml PBS 7 days prior to use. Thymus, bone marrow and spleen cells were properly dispersed in Eagle's minimal essential medium (MEM) as previously reported (8). Cells were then washed, counted by means of eosinophil counting chambers, mixed with the DNP-SRBC antigen where appropriate and injected into a lateral tail vein. Complete details of these procedures are described elsewhere (8).

Glass and nylon wool column depletion. Glass and nylon wool depletion of adherent cells and B lymphocytes from spleen cell suspensions was carried out as described by Julius *et al.* (9). Briefly, plastic syringes were packed with glass or nylon wool and

washed with medium (5% fetal calf serum in MEM) before use. Cells in a 2 or 3 ml volume were then poured into the glass wool column and immediately eluted with medium. Following centrifugation and resuspension the cells in 2 ml medium were loaded onto a nylon wool column and immediately eluted with medium. Following centrifugation and resuspension the cells in 2 ml medium were loaded onto a nylon wool column which had been preincubated in a humidified incubator for 1 hr at 37°C. Cells were eluted with warm medium after 45 min incubation at 37°C and washed twice with MEM (without fetal calf serum) prior to use.

Antisera. Anti-brain associated theta (anti-BA-Thy 1) serum was prepared by giving rabbits at a one month interval 2 intramuscular injections of a CBA mouse brain-complete Freund's adjuvant emulsion (10). Subsequent booster injections were given one week prior to each bleeding. Collected sera were absorbed with BC3 $F_1$ mouse liver and agarose, then titrated by measuring cytotoxicity against mouse thymocytes. Serum used in these experiments showed >95% killing of $10^6$ cells at a 1:200 dilution. Anti-mouse immunoglobulin prepared in goats was obtained from Cappel Laboratories (Downington, PA).

Antiserum treatment of cells. To a washed single cell spleen suspension was added 0.5 ml/spleen of antiserum, 4 ml MEM and sufficient complement to yield a final 1:10 dilution. The mixture was incubated 45 min at 4°C and washed twice with MEM prior to quantitation and use.

Plaque forming cell (PFC) assay. Spleen cells were assayed by the Jerne hemolytic plaque assay as modified for use with agarose gel, details of which are described by Miller and Cudkowicz (8). Guinea pig complement (Suburban Serum Labs, Silver Springs, MD) was used to develop direct plaques and rabbit anti-mouse γ-globulin (Cappel Laboratories, Downington, PA) was used for indirect plaque development at a dilution inhibitory for 90% of direct PFCs.

## RESULTS

PFC inhibition by whole spleen and T enriched spleen but not serum. The accompanying paper has demonstrated the ability of DNBS primed spleen cells to suppress PFC response to SRBC upon adoptive transfer and DNP-SRBC challenge of recipients. The results of two experiments included in the preceding report indicated that suppression was at least partly abrogated by treatment of donor cells with anti-Thy 1 serum prior to adoptive transfer. Substantiating this finding of T dependent mediation are the results of an experiment depicted by Figure 1. Paired groups of normal recipients (A-B, C-D, E-F) were given either $1.5 \times 10^7$ normal or DNBS primed spleen cells, $1.5 \times 10^7$ normal or DNBS primed spleen cells enriched for T cells by glass and nylon wool column elution, or 0.2 ml serum from normal or DNBS primed donors. These recipients (simultaneously challenged with DNP-SRBC) were assayed for anti-SRBC PFCs 5 days later. A comparison of normal and DNBS primed whole spleen recipients revealed that PFC responses of the latter group were inhibited in both the IgM (NORM:$408 \times 10^3$; DNBS:$342 \times 10^3$) and IgG (NORM:$296 \times 10^3$; DNBS:$222 \times 10^3$) assay. As shown by the responses of groups C

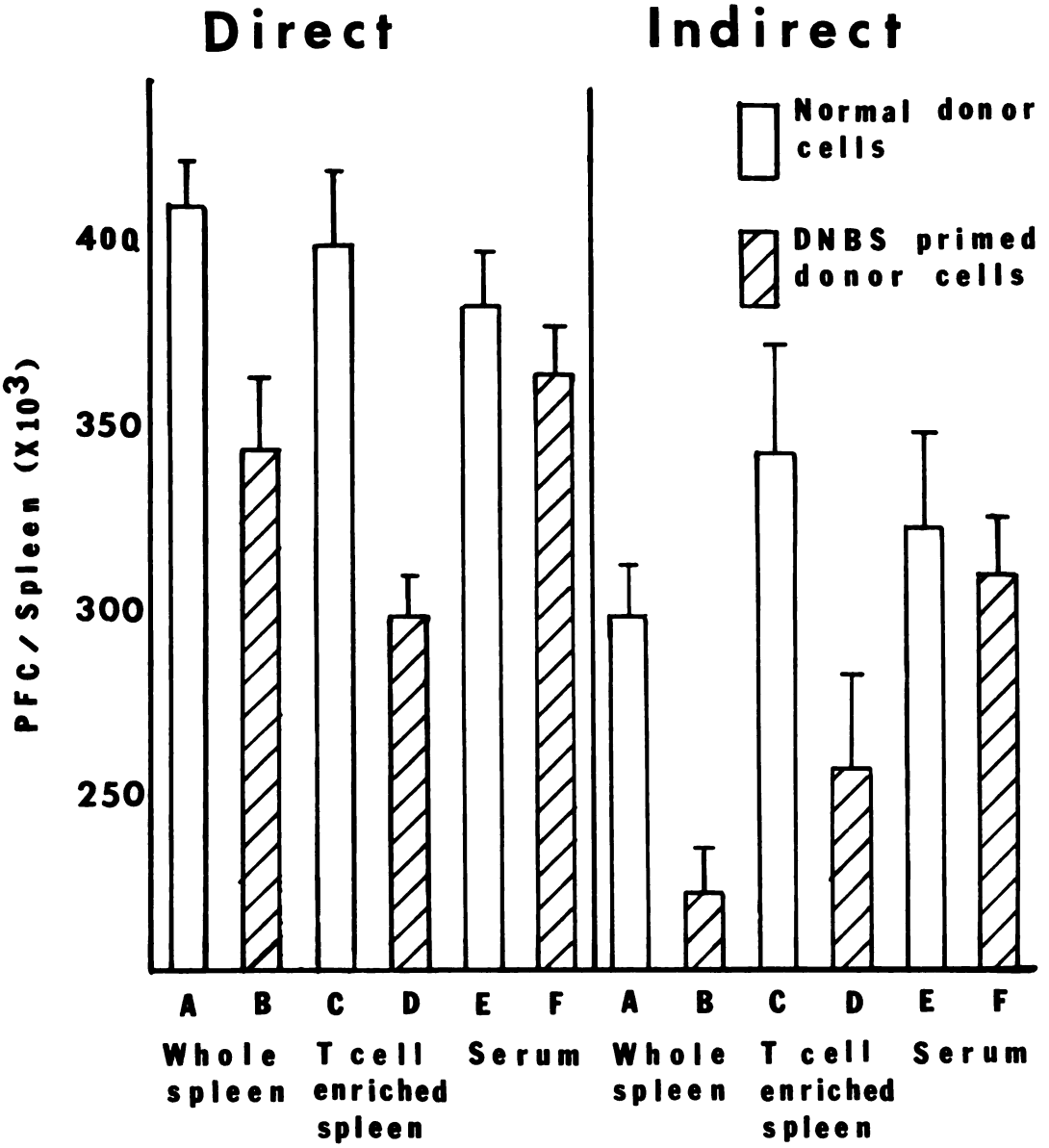


Figure 1. Responses obtained upon the transfer of whole spleen, T enriched spleen, or serum from normal or DNBS primed donors. Recipients were given 1.5 x $10^7$ cells or 0.2 ml serum as well as a DNP-SRBC antigen challenge on day 0 and were assayed on day 5. Statistically significant p values obtained by comparison of the experimental and control group in a 1 tailed Student t test are: direct AB<.025, CD .01; indirect AB<.005, CD<.005. Response inhibition was calculated by the formula:

$$\frac{\text{Norm PFC} - \text{DNBS PFC}}{\text{Norm PFC}} \times 100.$$

and D, enrichment for T cells and consequent depletion of B cells prior to transfer did little to alter this suppression. PFC inhibition was apparent in both the direct (NORM:397 x $10^3$; DNBS $\pm$ 296 x $10^3$) and indirect (NORM:310 x $10^3$; DNBS:251 x $10^3$) assay. In addition to reaffirming the T dependent nature of this activity, the results also agree with the earlier finding that suppression does not increase linearly with the addition of more donor cells since the enriched population contained proportionately more T cells. The paired groups which received serum from normal or DNBS primed donors showed no statistically significant PFC suppression in either the direct (NORM:381 x $10^3$; DNBS:362 x $10^3$) or indirect (NORM:320 x $10^3$; DNBS:306 x $10^3$) phases of response.

Suppression in x-irradiated and reconstituted recipients. Preceding experiments have demonstrated PFC suppression in immunologically intact (or nearly intact) recipients. To determine whether or not the same effect can be produced in lethally irradiated (immunologically void) recipients upon rescue with relatively pure populations of T and B cells, several experiments were completed. Table 1 summarizes the results of two such experiments. In the first, x-irradiated animals were given $10^7$ thymus cells and 15 mg DNBS, with normal controls foregoing the DNBS injection. One week later all animals received $10^7$ B cells (from donors which had been thymectomized, irradiated, and bone marrow reconstituted) and a DNP-SRBC challenge. Individual direct responses of the DNBS educated group and the control group varied considerably. While an apparent difference in PFCs/spleen did exist (NORM:1350; DNBS:790), this difference, because of a

Table 1. Suppression of PFC response in irradiated recipients by DNBS educated thymus or spleen T cells

| Experiment and Group | Protocol Day -7 | Protocol Day 0 | Anti-SRBC PFC/spleen + SE[a] Direct | Anti-SRBC PFC/spleen + SE[a] Indirect |
|---|---|---|---|---|
| I[b] A | $10^7$ thymus | $10^7$ B | 1350 ± 540 | 110 ± 17 |
| B | $10^7$ thymus and DNBS | $10^7$ B | 790 ± 186 | 47 ± 8 |
| II[c] C | | $10^7$ spleen T (Norm) + $10^7$ B | 1076 ± 111 | 254 ± 22 |
| D | | $10^7$ spleen T (DNBS) + $10^7$ B | 696 ± 102 | 112 ± 20 |

[a] p values of significant differences as determined by a 1 tailed Student t test in a comparison of the test group and its control group are: direct CD<0.025; indirect AB<0.010, CD<0.005. Assays were done on day 6.

[b] Nine mice/group were lethally irradiated and given $10^7$ thymus cells. Group B additionally received 15 mg DNBS. Seven days later both groups were given $10^7$ B cells and a DNP-SRBC challenge. B cells were prepared in irradiated, thymectomized, bone marrow repopulated animals.

[c] Six mice/group were injected with $10^7$ T and $10^7$ B lymphocytes as well as DNP-SRBC following lethal irradiation. T cells were prepared from normal or DNBS primed donors by treatment of spleen cells with rabbit anti-mouse Ig and complement. B cells were prepared by treatment of normal F, spleen cells with anti-BA-Thy 1 serum and complement.

great variability among individuals, did not prove statistically significant. However, the difference of the mean indirect response of the normal group (110 PFC/spleen) and the DNBS primed group (47 PFC/spleen) did prove statistically significant.

Several modifications of this protocol were incorporated into the design of the second experiment presented in Table 1. Normal or DNBS primed spleen cells, depleted of B cells by treatment with anti-mouse Ig antiserum were substituted for thymocytes as a source of T cells. B cells were prepared from normal spleens by depletion of Thy 1 positive cells with antiserum and complement. Irradiated recipients were given the T cell, B cell, and DNP-SRBC challenge component simultaneously and assayed five days later. The results again show that in relation to control levels PFC values in both the direct (NORM:1076; DNBS:696) and indirect (NORM:254; DNBS:112) responses were inhibited.

Shown in Figure 2 are the results of an experiment of similar design. Four groups of x-irradiated recipients were given $10^7$ B cells prepared in thymectomized, irradiated and bone marrow reconstituted donors. In addition to a DNP-SRBC challenge, each of the groups received one of the following types of spleen cells: normal, DNBS primed, anti-Thy 1 treated normal, or anti-Thy 1 treated DNBS primed. The results indicated that in comparison to control group A, the mean response of group C which received DNBS primed spleen cells as a source of T cell activity was strongly inhibited in indirect PFCs (NORM:442; DNBS:268) while less so in direct PFCs (NORM:1120; DNBS:962). Mean responses of groups B and D, transplanted with anti-BA-Thy 1 treated normal or DNBS primed spleen cells in addition to B cells, were each found to be in the range of 100 and 15 PFCs per spleen for direct and indirect responses, respectively. This showed that in the absence of T cell activity DNBS priming had no effect on relative responses. Furthermore, the

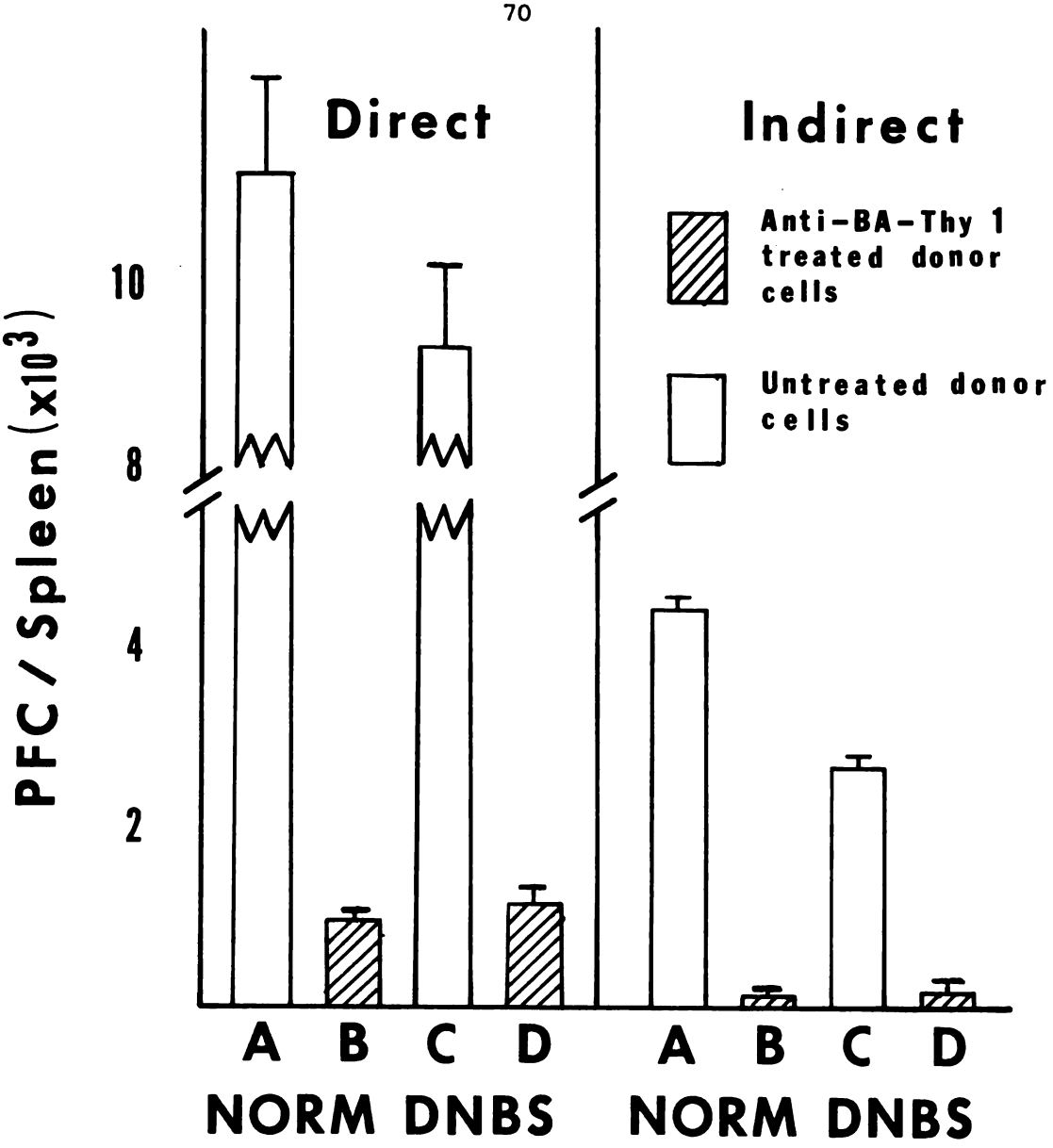


Figure 2. Suppression of PFC response by DNBS treated T cells in B repopulated, irradiated recipients and lack of a response in the absence of T cells. B cells ($10^7$) from irradiated, thymectomized, and bone marrow repopulated animals were given to each group of mice and followed one week later with $10^7$ normal (A), DNBS (C) or anti-Thy 1 treated normal (B) or DNBS (D) spleen cells in addition to a DNP-SRBC challenge. The assay was performed on day 6 and groups consisted of 8 mice with the exception of group C which included 7. Statistically significant p values obtained by comparison of the experimental group and its control group in a 1 tailed Student t test are: direct AB, CD<.001; indirect AC<.05, AB, CD<.001.

near elimination of responses in the absence of T cells indicated that the anti-SRBC response measured in these experiments was truly T dependent.

Allogeneic graft vs. host stimulation of DNBS primed recipients. We have, to this point, been concerned with defining the general properties of DNBS induced suppression. It was recognized that one possible means of further characterizing this T dependent suppression lay in establishing its relationship to a second, more studied T dependent activity. Table 2 summarizes the experimental design and results of one experiment in which suppression and the enhancement seen in allogeneic stimulation were allowed to compete in a

Table 2. Effect of allogeneic stimulation on the PFC response of DNBS primed recipients

| Recipients[b] | Priming | Donor Cells | Anti-SRBC PFC/spleen ($\times 10^3$) + SE[a] Direct | Indirect |
|---|---|---|---|---|
| A BC3 $F_1$ | none | C3H | 600 ± 52 | 442 ± 44 |
| B BC3 $F_1$ | DNBS | C3H | 560 ± 35 | 314 ± 22 |
| C BC3 $F_1$ | DNBS | $F_1$ | 292 ± 17 | 170 ± 16 |

[a] p values of significant differences determined by 1 tailed Student t test: Direct AC<0.005; indirect AB<0.025, AC<0.001.

[b] Groups contained 10 animals each. Animals in groups B and C were primed by 15 mg DNBS 7 days prior to transplantation of donor cells and administration of the DNP-SRBC challenge. Animals were assayed 5 days after receiving the donor cells and antigen.

response. Mice of the BC3 $F_1$ strain used in the previous experiments were employed as recipients (nonirradiated) in the following manner. While group A served as an unprimed control group, groups B and C were primed with DNBS on day -7. At day 0 all three groups were transplanted with $1.5 \times 10^7$ donor spleen cells consisting of parental type cells for groups A and B, and sygeneic cells for group C. At the same time each group was challenged with DNP-SRBC. Allogeneic stimulation resulted in an indirect response of $442 \times 10^3$ PFC/spleen for control group A and $314 \times 10^3$ PFC/spleen for DNBS primed group B. Group C which was also DNBS primed but received syngeneic instead of allogeneic cells produced only $170 \times 10^3$ PFC/spleen. The corresponding values for the direct assay were $600 \times 10^3$, $560 \times 10^3$, and $292 \times 10^3$ PFC/spleen, respectively. These results suggested that while allogeneic stimulation resulted in a doubling of both the IgM and IgG response over normal levels at least in the IgG phase, the relative level of suppression resulting from DNBS priming is maintained.

To further examine DNBS induced suppression in competition with nonspecific allogeneic stimulation, the following protocols were employed. The first experiment described in Table 3 utilized four groups of recipients. Groups B-D were primed with DNBS on day -7 and group A served as an unprimed control. On day 0 each group received a DNP-SRBC challenge in addition to $10^7$ spleen cells of the type indicated in the following scheme: A and B - C3H, C - lethally irradiated C3H, and D - syngeneic $F_1$. A comparison of PFC/spleen in normal and DNBS primed allogeneically stimulated animals revealed that inhibition occurred in both the direct (NORM:$368 \times 10^3$; DNBS:217

Table 3. Suppression of PFC response by host but not graft cells upon allogeneic graft vs. host stimulation

| Experiment and Group | Recipient[b] Status | Donor Cells | Anti-SRBC PFC/spleen ($x10^3$) + SE[a] Direct | Indirect |
|---|---|---|---|---|
| I A | Norm | C3H | 368 ± 50 | 42 ± 3 |
| B | DNBS | C3H | 217 ± 12 | 30 ± 3 |
| C | DNBS | C3H (irrad)[c] | 96 ± 10 | 24 ± 2 |
| D | DNBS | $F_1$ | 141 ± 13 | 22 ± 2 |
| II E | Norm | C3H (Norm) | 495 ± 35 | 88 ± 14 |
| F | Norm | C3H (DNBS) | 437 ± 31 | 67 ± 4 |
| G | Norm | C3H (DNBS,anti-Thy 1)[d] | 98 ± 15 | 61 ± 6 |
| H | Norm | C3H (DNBS,anti-Ig)[d] | 808 ± 47 | 117 ± 13 |

[a] p values of significant differences between groups as determined by a 1 tailed Student t test: direct AB<0.025, BD<0.005, BC, FG and FH<0.001; indirect BC and BD<0.05, AB and FH<0.010.

[b] DNBS indicates recipients (or donors) were treated with DNBS 1 week prior to injection of donor cells (day -7). All animals were challenged with DNP-SRBC on day 0 and assayed on day 5.

[c] Cells were lethally irradiated (950 rads) *in vitro* just prior to transfer.

[d] Donor cells for groups G and H were treated *in vitro* with the indicated antisera prior to transfer.

x $10^3$) and indirect (NORM:42 x $10^3$; DNBS:30 x $10^3$) responses. Direct and indirect PFC/spleen produced by group C which received cells inactivated by radiation (96 x $10^3$ and 24 x $10^3$, respectively) do not differ greatly from those values observed in group D following transfer of syngeneic instead of allogeneic cells (140 x $10^3$ and 22 x $10^3$). As in the previous experiment the allogeneic stimulus greatly enhanced responses yet preserved the relative levels of suppression seen upon comparison of normal and DNBS primed animals. The responses of groups C and D indicate that the allogeneic stimulus in this system is a product of the donor cells. Since only the recipient's cells in the appropriate groups were primed by DNBS, this experiment implies that the responding B cells were influenced by two separate T dependent messages, though both may not necessarily have acted at the same time. In this and the following experiment the low indirect response values were due to a particular lot of anti-immunoglobulin antiserum which proved inefficient in developing indirect PFCs.

The aim of the second experiment in Table 3 was to determine whether or not cells derived from one source could supply both the allogeneic as well as the suppressive signal simultaneously. Primed spleen donor cells were prepared in C3H mice by administration of DNBS one week prior to sacrifice. Each of four groups of $F_1$ recipients were injected with $10^7$ viable donor cells according to the following scheme: group E received normal C3H cells, F received DNBS primed C3H cells, G received anti-Thy 1 treated DNBS primed C3H cells, and H received anti-mouse Ig treated DNBS primed cells. The differences in PFC responses of the control and DNBS test groups were small in both the direct (NORM:495 x $10^3$; DNBS:437 x $10^3$) and indirect (NORM:

88 x $10^3$; DNBS:67 x $10^3$) assay and not statistically significant, suggesting the suppressive signal in this situation is either masked or nonexistent. The PFC responses of group G, which received T depleted donor cells, were low (direct:98 x $10^3$; indirect:61 x $10^3$) and approximated those of group C above which had received inactive irradiated cells. This again indicated that all of the allogeneic stimulus came from the T cell fraction of the C3H cells. The PFC response of group H which received T enriched (B cell depleted) C3H cells was nearly double that of group F which was given C3H whole spleen (H - direct:808 x $10^3$; indirect:117 x $10^3$ as opposed to G - direct:437 x $10^3$, indirect:67 x $10^3$). It appears that the higher ratio of T cells in this population resulted in a proportionately greater amount of allogeneic stimulus.

Inability of parental type cells to suppress $F_1$ responses. A more critical test of the capacity of one cell population to provide enhancement and yet maintain a characteristic level of suppression is shown in Table 4. Two paired groups of lethally irradiated $F_1$ recipients were given either $10^7$ normal or DNBS primed T cells of $BC3F_1$ or C3H origin and concurrently $10^7$ $BC3F_1$ B cells as well as a DNP-SRBC challenge. Comparison of mean PFC/spleen in the recipients which were given the syngeneic T cells reveals that strong suppression occurred at the direct (NORM:1076; DNBS:696) as well as indirect (NORM:254; DNBS:112) level. Thus, while the allogeneic T cells provided considerable overall PFC enhancement in the other two groups statistically significant suppression was seen in neither the direct (NORM:2691; DNBS:3058) nor indirect (NORM:389; DNBS:336) response.

Table 4. Failure of parental T cells to suppress $F_1$ B cell responses in lethally irradiated hosts

| Group | Donor Cells | Anti-SRBC PFC/spleen + SE[a] Direct | Indirect |
|---|---|---|---|
| A | $10^7$ $F_1$ T (Norm)<br>$10^7$ $F_1$ B | 1076 ± 111 | 254 ± 22 |
| B | $10^7$ $F_1$ T (DNBS)<br>$10^7$ $F_1$ B | 696 ± 102 | 112 ± 20 |
| C | $10^7$ C3H T (Norm)<br>$10^7$ $F_1$ B | 2691 ± 373 | 389 ± 24 |
| D | $10^7$ C3H T (DNBS)<br>$10^7$ $F_1$ B | 3058 ± 334 | 336 ± 48 |

[a] p values of significant differences as determined by 1 tailed Student t test in a comparison of the test group and its control are: direct AB<0.025; indirect AB<0.005.

[b] Host animals were lethally irradiated and six mice/group were given the indicated lymphocytes and a DNP-SRBC challenge. T cells were prepared from normal or DNBS treated $F_1$ or C3H donors by treatment of spleen cells with rabbit anti-mouse Ig and complement. B cells were prepared by treatment of normal $F_1$ spleen cells with anti-BA-Thy 1 serum and complement. The assay was performed on day 6.

Suppression of parental response by DNBS primed $BC3F_1$ cells.

The experiment represented by Table 5 designed to determine whether or not $F_1$ cells are capable of suppressing the response of a parental type host under host vs. graft conditions. One group of normal parental C3H recipients was given $10^7$ normal spleen cells while a second was given $10^7$ DNBS primed $BC3F_1$ cells. Both groups were

Table 5. Suppression of parental host PFC response by $F_1$ DNBS-primed donor cells

| Recipients[b] | Donor Cells | Anti-SRBC PFC/spleen ($x10^3$) + SE[a] Direct | Indirect |
|---|---|---|---|
| A C3H | $F_1$ (Norm) | 543 ± 30 | 758 ± 15 |
| B C3H | $F_1$ (DNBS) | 438 ± 15 | 663 ± 16 |

[a] p values of significant differences as determined by a 1 tailed Student t test are: direct AB<0.025; indirect AB<0.005.

[b] Normal recipients were given $10^7$ viable donor cells in addition to a DNP-SRBC challenge 5 days prior to assay.

challenged with DNP-SRBC and assayed five days later. It was found that the DNBS primed cells had effected suppression of the host's antibody producing cells in the direct (NORM:543 x $10^3$; DNBS:438 x $10^3$) as well as indirect (NORM:758 x $10^3$; DNBS:663 x $10^3$) phase. This suppression, though modest, implied that C3H antibody forming cells were influenced by $F_1$ suppressor cells. Suppression, superimposed on the high level of response, once again indicated that antibody producing cells were influenced by both an enhancing and suppressing signal.

## DISCUSSION

In the accompanying paper DNBS treatment of donor $BC3F_1$ mice was shown to induce DNP specific suppressor cells in the spleen. Upon adoptive transfer such cells were shown to reduce the antibody response of recipients challenged with DNP-SRBC. It was noted that PFC inhibition, routinely achieved by the transfer of 10-20 x $10^6$

cells, does not increase linearly with the transfer of more cells, occurs in secondary as well as primary responses and is inhibitable by anti-BA-Thy 1 antiserum. The experiments in this paper are an extension of this work and have as their purpose a more complete characterization of this system.

The major findings relating to this objective can be collectively presented as follows: 1) While for most systems studied to date suppressor cells were found to be recruited from the recipient's lymphoid population (11), suppressor cells in the current study seemed to be directly induced in the donor population. B cell repopulated, x-irradiated recipients given relatively pure population of DNBS primed thymus or splenic T cells showed suppressed responses when compared with recipients given normal cells of the same type. 2) The suppressive activity resides in cells but not serum since the response of recipients given serum from DNBS primed donors was no different than that of recipients given normal serum. 3) Equal numbers of DNBS treated unfractionated or T enriched spleen cells provide a comparable degree of suppression in normal recipients. This reinforced the findings of an earlier experiment which indicated that this particular type of suppressor activity is conveyed by T cells and the availability of proportionally more T cells (in an enriched population) does not result in greater suppression. 4) The suppressing signal is expressed when competing with a nonspecific allogeneic enhancing signal also produced by T cells. This was shown in several experiments in which either DNBS primed animals or B repopulated, x-irradiated recipients given DNBS primed T cells received a source of allogeneic signal. The allogeneic boost was

provided by parental cells reacting against hybrid $F_1$ cells. 5) In either whole or irradiated $F_1$ animals allogeneic enhancement and proportionate suppression occurred when $F_1$ but not parental cells were DNBS primed. It was shown that cells of one source ($F_1$ or parental) did relay both the allogeneic enhancing signal and the specific suppressing signal simultaneously. $F_1$ DNBS primed cells, however, as the allogeneic target of parental cells, were still capable of providing the suppressive signal.

Mechanistically, direct induction of suppressor cells is not necessarily incompatible with other reported suppressor phenomena. Rather, this feature may reflect some property of the inducing determinant group which allows only incomplete stimulation. DNP sensitization in fact seems to favor the induction of T cells for a cellular type of reactivity (12). Even with DNP-protein conjugate sensitization attempts to show DNP induced helper activity against the protein moiety of the conjugate have been only marginally successful (3,4,5). It is then not inconceivable that this predilection toward delayed responses and this inability to induce effective helper activity may be related to suppression inducing properties. In addition to our own study Phanuphak *et al.* (13) have described a situation in which the adoptive transfer of DNBS primed spleen or lymph node cells to recipient mice prevented the development of DNP-specific contact sensitivity in these animals. This situation may then be similar to one described by Kapp *et al.* (14) in which mice genetically unresponsive to the random terpolymer L-glutamic acid$^{60}$-L-alanine$^{30}$-L-tyrosine$^{10}$ (GAT), when confronted with this antigen in the free form, developed suppressor cells which inhibited

subsequent GAT antibody response after challenge by GAT conjugated to an immunogenic carrier.

The nature of the suppressive signal remains uncertain. The absence of activity from the serum of DNBS primed donors considerably reduces the possibility that antibody is involved. Yet it does not rule out mediation of suppression by a soluble factor. Absence of factor activity in the serum at the time of cell transfer might be explained if such a factor were short-lived or labile and without restimulation of T cells by either antigen or the trauma of transplantation its production stops. Such lability is consistent with the findings of Tada (15), who reported that the activity of the suppressive factor extracted from mechanically disrupted thymocytes or spleen cells is unstable and persists only a short time.

The allogeneic effect, reported by Osborne and Katz (16), results in a nonspecific boost in antibody response against the antigen to which the animals were previously immunized. Armerding and Katz (17) have recently characterized this activity as a T cell produced allogeneic effect factor (AEF), found in T cell supernatant following allogeneic stimulation. We have placed this T cell factor mediated effect in direct competition with T cell mediated suppression to establish the nature of the relationship between the two effects. At the present stage these experiments suggest that two signals are operating in this encounter, one effecting an overall nonspecific doubling of responses and the other preserving the proportionate suppression that was observed when responses of DNBS primed and normal mice were compared. This has a number of implications for T cell regulation of B cells.

On the basis of these experiments it cannot be determined whether the two signals are distinct or share a common structural component. Successful adaptation of this type of experiment to *in vitro* conditions might permit identification of the suppressor signal by the use of defined antisera. The fact that the suppression was antigen specific suggests an association with the T cell receptor. No specificity has yet been linked with the allogeneic effect and hence association of this factor with the antigen receptor is unlikely. AEF was found by Armerding and Katz (17) to be sensitive to antisera directed against Ia and H-2 but not Ig cell determinants.

In the past a number of models have been proposed to explain T cell participation in antibody response and in one respect or another each has been hard pressed to account for various data. More recent models are basically variations of a theme ascribing what is now referred to as the "spectrum" of helper to suppressor activity to either a single type of T cell or to two or more T cell subpopulations (11). The major difficulty usually encountered in one cell theories is how this cell, presumably with one type of antigen receptor, translates a particular antigen encounter to an appropriate regulatory signal. Katz and Benacerraf (18) have proposed an elaborate model in which the differentiation state of the T cell and several T cell products determine this matter. In their scheme all T lymphocytes at an immature stage express an antigen receptor coded for within the K or I region of the H-2 complex. Those cells must also express a cell interaction (CI) molecule, possibly the Ia gene product, before cooperation with cells bearing a similar marker or complementary receptor can occur. Interaction

with antigen at this immature stage leads to suppression. Helper activity requires expression of Ir gene product in addition to the other two early products and comes only with maturation. The Ir product modifies the antigen receptor by some type of molecular association. Nonspecific AEF in this scheme is a complex of the Ir and CI products. The antigen specific suppressive activity we have studied, in terms of this model, would consist of the CI-antigen receptor complex found on incompletely differentiated cells. If this model is basically correct it could be predicted that this suppression is sensitive to anti-Ig but not anti-Ir or anti-Ig sera. Anti-H-2 but not anti-Ig sensitivity has in fact been found for an antigen specific suppressing factor by Tada (15).

Less attractive at present is the alternative model which postulates the existence of two T cell types to account for help and suppression. The acceptance of this model is more difficult because at present it cannot explain certain results, e.g., how the removal of one fraction of T cells can aid response in one instance and depress response in another (19). The two cell theory could be compatible with findings of the present study in the simultaneous presence of the enhancing and suppressing signals. However, this line of argument is weakened by the observation that for simultaneous expression of the enhancing and suppressing factors to occur in our system these activities had to be conveyed independently by the $F_1$ and parental cell types.

This study has addressed one aspect of regulation by examining DNBS induced suppression. While the mechanism and level at which suppressor cells act remains obscure, this study suggests that T cells

can solely account for this suppression and the suppressive signal can compete with a second type of T cell signal. The DNBS system modified for use in conjunction with other antigens to improve the relative level of suppression could prove extremely valuable in further mechanistic studies.

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# APPENDIX

# APPENDIX

## Introduction

Lymphocytes, as one of the most functionally diverse cell populations known, are also one of the most studied. However, with regard to fundamental cellular events, our knowledge is incomplete. Scanning electron microscopy (SEM), already a powerful research tool in a number of fields, has great potential in closing some of these gaps (1). To date, however, its use in immunology has been largely limited to the morphological characterization of lymphoid cells (2,3,4). Development of a reliable immunolabeling technique is necessary to allow routine use of this instrument for such problems as heterogeneity in lymphocyte receptor binding, identification of lymphocyte classes and subclasses in cell interactions, differentiation characteristics of lymphocytes, quantitation of reactive lymphocytes as well as broader problems in other types of tissues. Development of such a technique is currently feasible because there are already available a number of antisera species- or individual-specific for cell surface determinants. Current research literature contains a number of reports describing a variety of visual marker particles and coupling techniques by which these antisera could be used (5,6). A good immunological marker has two primary requirements: it must be recognizable at low survey magnifications and it must exhibit a

high degree of binding specificity. The techniques described to date have been found wanting in one or both of the criteria.

The project described in this section, carried on in conjunction with the foregoing study, has as its objective the development of a practical and reliable immunolabeling technique suitable for use with SEM. Visual markers available for this technique include biological materials such as viruses, bacteriophages and large molecular weight proteins or nonbiologicals such as synthetic polymers (4,5,6). Cell surfaces and receptors bind or are bound nonspecifically by many of these markers because of their surface properties. This study was encouraged by the recent development of a polyacrylamide particle said to eliminate nonspecific sticking to target cells (7). In addition, it can be synthesized inexpensively in various sizes in the laboratory and each particle has available numerous carboxyl and hydroxyl groups for covalent attachment of antibody or antigen molecules to give it specificity in binding.

## Methods

### Cell Preparation

The early phase of this research was concerned primarily with the development of preparative procedures. This involved modifying and combining portions of existing procedures (1,3,5,7) as well as considerable improvisation. The drying and fixing of thymus, spleen and bone marrow cells is done as follows. Single cell suspensions in 1% glutaraldehyde (adjusted to 280 m osmol) are incubated for 1 hr at 25°C and overnight at 4°C. An aliquot of these cells (2-4 x

$10^6$) is then aspirated onto 13 mm nucleopore membranes in a specially devised holder and washed with phosphate buffered saline (PBS). The fixed cell sample is then dehydrated successively for 5-min periods through 50, 75, 95 and 100% ethanol. Samples are critical point dried, mounted on aluminum stubs, and coated with 200 Å gold prior to examination by SEM.

Preparation of Labeling Materials

The antisera employed were those used in the investigation of DNP sensitive regulatory cells (see Materials and Methods of accompanying papers). Antibody for latex coupling was prepared by ammonium sulfate precipitation of immunoglobulins from the relevant antisera and subsequent lyophilization. Distillation of methacrylate monomers, synthesis of spheres, and deionization of the spheres is described by Molday *et al.* (7). The procedure for coupling molecules to the markers, also described by Molday *et al.* (7), was modified in several respects. Initially 150 mg of latex was derivatized with diaminoethane or ε-amino-caproic acid (5 ml total volume of a 0.01 M solution) by the addition of 20 mg carbodiimide (7,8). Following 2 hr of stirring at 4°C the mixture was dialyzed against 0.1 M NaCl. Antibody was covalently bound to the amino groups of the diamino-derivatized latex spheres by means of a second carbodiimide reaction. Twenty milligrams carbodiimide, 10 mg of antibody and 50 mg of latex in 2 ml total volume at pH 7 was stirred for 2 hr at 4°C. The reaction was stopped by the addition of 0.1 M glycine at pH 8 and the latex washed three times in saline.

Glutaraldehyde coupling of antibody to latex was carried out in two steps. First aqueous glutaraldehyde, yielding a final 2.5% concentration, was added to a suspension of diaminoethane derivatized latex (150 mg) in 0.01 M sodium phosphate buffer (5 ml total vol). After 4 hr at 25°C the mixture was dialyzed to remove excess glutaraldehyde. Ten milligrams of antibody was then added to 50 mg activated latex in phosphate buffer (2 ml total vol) and the suspension shaken for 3 hr at 25°C. Excess antibody was removed by centrifugation and washing.

DNP labeled latex was prepared by mixing 50 mg of diaminoethane derivatized latex, 3 mg ε-DNP-aminocaproic acid and 20 mg of carbodiimide at pH 6 (total vol of 2 ml) for 2 hr at 25°C. The reaction was stopped by washing with saline. Both this preparation and the antibody bound latex was resuspended in saline and stored at 4°C.

Immunolabeling Techniques

For direct immunolabeling, markers are given specific reactivity by the attachment to antibody directed against a target determinant. In this study direct labeling was carried out using rabbit anti-Thy 1 or anti-mouse Ig as the antibody component. Tissue adsorbed normal rabbit serum antibody was used as a control preparation. Target cells were washed twice and resuspended in PBS at $10^7$ cells/ml. A mixture of 0.1 ml antibody coated latex and 0.5 ml cell suspension was then incubated at 25°C for 30 min. Following removal of the excess latex by low speed centrifugation, cells were fixed as described above.

Indirect techniques require specific reaction of the target and free specific antibody as a first step and then secondarily employ reaction with a species specific anti-immunoglobulin coated marker particle. The current study used rabbit anti-Thy 1 and anti-mouse Ig in the former role and anti-rabbit Ig coated latex as the marker-bound antibody. Reaction conditions were similar to those used in direct labeling, with excess antibody washed out prior to the second 30-min incubation period.

Experiments to detect antigen binding cells were carried out by incubating DNP derivatized latex with DNP immunized splenic lymphocytes (see Materials and Methods of the accompanying papers). Cell and latex concentrations and the conditions of reaction are similar to those described for direct and indirect labeling.

## Results

From several batches of latex synthesized, particles of 150 and 280 nm diameter were chosen for the immunolabeling experiments. The first phase of this study tested an indirect technique in which the markers are coupled to goat anti-rabbit Ig antibody. These markers are then reacted with target cells already exposed to rabbit antiserum specific for a target determinant. Target cells consisted of thymus or spleen lymphocytes and the specific antiserum used was anti-mouse Ig.

Results obtained with different batches of antibody coupled latex were variable. Mainly affected was the degree of background binding by latex. In control preparations preincubated with normal rabbit serum (NRS), background ranged from 0 spheres per cell in

some trials to 5 or more spheres per cell in others. The use of higher or lower concentrations of latex (keeping constant the concentration of the determinant specific antibody) in general correspondingly raised or lowered background labeling. Interpretation of specific labeling with regard to the control was often difficult. Figure 1 depicts the results of one experiment employing Thy 1 labeling. Micrographs A and B show control and specific labeling respectively for thymus cells. Micrographs C and D similarly demonstrate this with spleen cell targets. The glutaraldehyde method of coupling antibody to latex was not found to improve the labeling efficiency.

The difficulty in reproducing results led to the testing of a direct labeling technique. This direct method inherently eliminates variables introduced by incubation of target cells with antiserum prior to labeling. Furthermore, the use of B cell specific anti-mouse Ig antibody bound to latex made it possible to employ a better control. By comparing the labeling efficiency of spleen samples which contain B cells and thymus samples which are relatively free of B cells, it was possible to avoid binding due to any nonspecific immunoglobulins in the NRS control preparation. Figure 2 depicts the results of one experiment in which thymus (micrograph A) and spleen (micrograph B) cells were so labeled. It is evident that a greater proportion of cells were bound by latex in the spleen sample. The results of other experiments were less clear. The preparation of anti-Thy 1-bound latex used in this particular trial showed little activity.

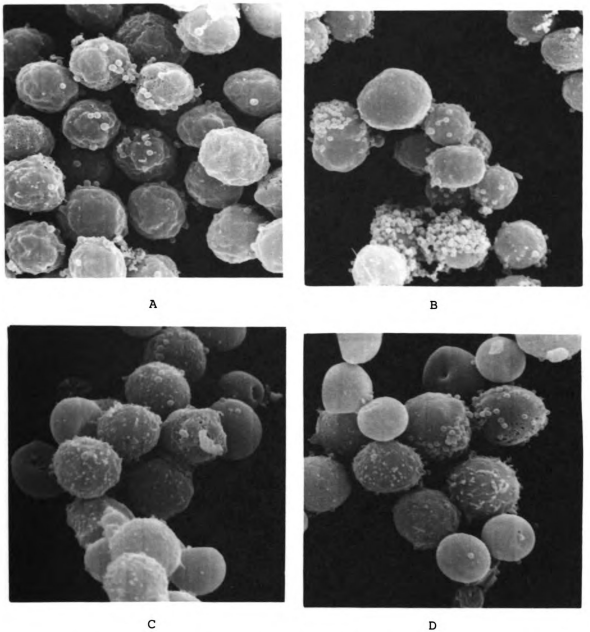
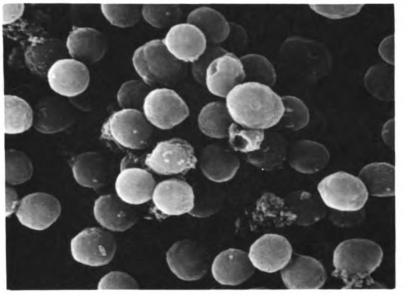
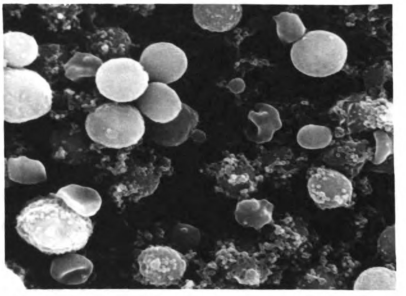



Figure 1. Indirect labeling procedure whereby normal mouse lymphocytes (5-7 μm in diameter) were incubated with NRS or rabbit anti-Thy 1 (T cell associated antigen) antiserum and subsequently treated with latex-bound goat anti-rabbit Ig antibody. Micrographs A and B show the labeling which results when thymus cells were pre-treated with NRS control preparation (A) or anti-Thy 1 antiserum (B). Micrographs C and D show the results of spleen pretreatment with NRS (C) or anti-Thy 1 antiserum (D) prior to labeling with anti-rabbit Ig-coated latex.



A



B

Figure 2. Direct labeling technique in which rabbit anti-mouse Ig antibody bound to latex was used to label cells having surface Ig (B cell associated antigen). Micrograph A shows the effect on a thymus preparation (lacking B cells) while micrograph B shows the effect on a spleen preparation (containing B cells).

The cell samples used for this project were obtained from the experimental animals used in the preceding study of regulation by DNP sensitized T cells. To determine the efficacy of labeling by antigen binding, a preparation of DNP bound to latex via a spacer molecule was incubated with cells. In several such attempts no preferential binding to certain cells within a population was evident. Regarding size of particles, the above experiments showed the 280 nm diameter spheres to be an ideal size for lymphocyte labeling because they remain distinguishable at survey magnifications while the smaller particles do not.

## Discussion

This study provided a valuable foundation for the further development of immunolabeling techniques. The experiments completed indicate that the nonspecific attachment of particles to target cells that occurs with the use of commercial latex is greatly diminished with methacrylate latex. Examination of latex labeled preparations containing contaminating erythrocytes revealed that the binding of latex particles to these cells is a relatively rare event. Background attachment to lymphocytes could then be explained by cross reactive or nonspecific binding on the part of the antibody or some contaminating protein moiety coupled to the particles give them specificity. This view is not incompatible with the results of the antigen binding experiments. DNP-derivatized particles, although not bound selectively by target cells through their determinant group as intended, did not nonselectively stick to lymphoid cells at a background level.

Experiments involving specific labeling have enjoyed some measure of success and further work may show that the methods used here contain the basis for a reliable procedure. The two major aims in further research of this type must be the achievement of reproducibility and the elimination of background. These problems may be greatly alleviated by the use of purified antibody preparations in which nonspecific antibody and other proteins are depleted and specific antibody activity is increased. This could also eliminate another type of background. In order to get sufficient binding in the experiments described above, it was necessary to use a relatively high concentration of latex. This often resulted in clumps of latex (formed as a result of storage) remaining on the sample and making interpretation difficult (see Figure 2).

Not often discussed in reports describing immunolabeling procedures is a problem peculiar to a successful technique. A target such as a lymphocyte has exposed on its surface a great number of antigenic determinants of any given specificity. Efficient labeling by antibody coated latex particles then results in the masking of the target cell surface features. The problem might be remedied by the use of a low concentration of high activity latex or alternatively the use of very large particles which are large enough to sterically limit the number of particles capable of binding any one cell. However, while a procedure which specifically and effectively coats target cells with latex may have limitations with regard to SEM, it may prove useful in yet other areas. For example, a latex coating would necessarily change the physical characteristics of a target. Cells of a given specificity within a population might then

be quantitated by means of a size discrminating cell counter or eliminated from a population by density or velocity centrifugation.

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