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FUNCTIONAL DIFFERENTIATION OF MEMORY B LYMPHOCYTES

by

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INTRODUCTION

I. Statement of the Problem.

B lymphocytes (bone marrow-derived, B cells) have a unique role in humoral immune responses in that they alone have the ability to produce antibody after antigenic stimulation. Although the nature of the activation signal(s) is poorly understood (see Ref. 1 for review of current concepts of B cell activation), two classes of antigens which can initiate these signals have been defined. 1) Those which require thymus-derived lymphocyte (T cell) help i.e. thymus-dependent (TD) antigens and 2) those which can activate B cells, more or less directly, without T cell help i.e. thymus-independent (TI) antigens. The latter antigens have as a common feature the ability to elicit predominantly the IgM class of immunoglobulin (2-6), although in certain instances IgG responses have been reported (7-12). The inability of TI antigens to trigger IgG synthesis has been ascribed to the relative T dependence of IgM and IgG secreting cells, since the latter are thought to be more T-dependent (2,12). However similar T-dependence has been observed for IgM and IgG secondary responses (13). Another common feature of TI antigens worth noting is that attempts to develop IgG memory B cells by priming with TI antigens has generally met with failure (4,5,9,14-22). When memory has been reported the priming effect has been 1) small (5,9), 2) observed only as increased IgM responses (5,14) or 3) seen only as a capacity to respond to doses of antigen which were lower than those needed for primary responses and thus non-immunogenic (17). It is curious that although memory B cells

can develop in the relative absence of T cells (23-25) no such memory can be generated by TI antigen forms.

Recently Braley-Mullen (26-27) reported *in vivo* secondary IgG responses to the TI antigen pneumococcal polysaccharide (SIII) subsequent to priming with the TD form of SIII, namely SII coupled to sheep or horse erythrocytes (RBC). Later (27) the secondary SIII IgG response was shown to be T-independent in that SIII-RBC primed spleen cells depleted of T cells responded equally well to SIII as did untreated primed cells in adoptive transfer. In contrast the ability to respond to the TD form, SIII-RBC, was eliminated in the T cell depleted spleens. This clearly indicated that in certain instances TI antigens can induce IgG antibody synthesis in the absence of T cells.

Braley-Mullen (26) also has shown that the ability to produce IgG responses by stimulation with SIII-RBC precedes the potential to do so with SIII alone and she has suggested the possibility that different memory cells may be involved in the responses to the two antigen forms (26).

Recent work from several laboratories indicates that the B lymphocytes responding to TD and TI forms of the same epitope in primary responses may represent distinct subpopulations of primary B cells (15,28-35). The ontogenetic relationship between these subpopulations is not known at this time. Such information is vital for our understanding of the developmental pathways which ultimately lead to a fully mature and functional B cell network.

The purpose of the work in this thesis is two-fold: 1) to develop an *in vitro* model for studying TI and TD memory IgG responses. 2) To

test the hypothesis that TD and TI-responsive IgG memory B cells constitute functionally distinct subpopulations as has been suggested for primary IgM responses.

II. Literature Review

A. Thymus-dependent and Thymus-independent Antigens.

As mentioned in section I above there are two classes of antigens which can trigger B lymphocytes to secrete Ig. One (TD) requires T cell help, the other (TI) does not require T cell help. What makes an antigen one or the other is not at all understood. One striking feature of TI antigens is they are all large polymers with repeating antigenic determinants (36), e.g. most polysaccharides are TI antigens and haptens coupled to these carriers also behave as TI antigens (36) suggesting that T independence is not related to the antigenic determinants of these immunogens per se but rather to their spatial array (36) and/or their susceptibility to degradation (10). Furthermore aggregation of TD antigens can lead to a TI form. Thus monomeric flagellin (MON) is TD but when aggregated (polymerized flagellin, POL) becomes TI (36), although a partial TD response is still suspected (37). Likewise, trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) is normally TD but when conjugated to sepharose beads, a carbohydrate backbone with many identical repeating units, the antihapten response becomes TI (38). Similarly, it should be noted that a TI antigen can be converted to TD by conjugation to a TD backbone (39). Dukor and Hartmann (40,41) have proposed that antigens which can cross-link surface Ig receptors would be TI if they could by themselves activate C3, the third component of

complement, and convey it to adjacent C receptor (CR) sites on the same membrane. Although there is some evidence that TI antigens can activate C3, the model becomes less appealing in light of recent data (15, 42-44) which show that the population of B cells responding to TI antigens lack CR prior to stimulation. However no information is available on whether the CR^- population could become CR^+ later. Coutinho and Moller have suggested that all TI antigens are mitogens (45) and thus can directly trigger B cells via a mitogenic signal. The hapten upon binding with surface Ig only facilitates the interaction of the mitogenic moiety with its specific receptor. Recently, it was found that spleen cells from one strain of mouse, C3H/HeJ, do not respond to the mitogenic influences of lipopolysaccharide (LPS, 46,47). Furthermore, these mice fail to respond to the TI antigen TNP-LPS (48,49) suggesting that the mechanism of hapten passively binding to surface Ig in order to focus mitogen to its receptor is indeed possible in some instances.

Recently TI antigens have been subdivided into two subclasses of TI antigens; TI-1 antigens trigger an immature population of B cells whereas TI-2 antigens activate a more mature population of B cells to secrete antibody (50-51). The reasons for this dichotomy are: 1) T-1 antigens can induce antibody responses in neonatal mice earlier than can TI-2 antigens (51,52), 2) TI-1 antigens can elicit a response in CBA/N mice (50,51) which have an X-linked B cell defect and do not respond to TI-2 type antigens (50,51,53), 3) they differ in their susceptibility to anti-δ mediated immunosuppression, with TI-2 antigens being more susceptible (54) and 4) TI-1 antigens give additive responses with TI-2 antigens but not with TD antigens (35).

Some examples of TD, TI-1 and TI-2 antigens are listed in Table I

and are referred to later in the text.

B. Ontogeny of B Cell Development.

In mice B lymphocytes have characteristic surface markers and functions that appear in sequence during development. In this section I shall present the various known surface markers and where possible relate these to TI and TD responsiveness.

1. Surface Immunoglobulin

The ontogenetic appearance of B lymphocytes bearing surface IgM ($sIgM^+$) in fetal liver is preceded by the development of a population of large lymphoid cells that contain small amounts of cytoplasmic IgM ($cIgM^+$) but lack sIgM detectable by immunofluorescence techniques (59, 60). These cells, termed pre-B cells, are present in fetal spleen, liver and bone marrow (61-63), but in adults are found exclusively in bone marrow (61,62,64) and development of these cells is apparently unaffected by chronic anti-mouse IgM (anti-μ) treatment (65). In fetal liver pre-B cells first appear around day 10-13 of gestation (66,67), while sIg^+ cells appear a few days later by the 14th or 16th day of gestation (68-70). These sIg^+ cells are found not only in liver but also in spleen and bone marrow. The developmental stages from pre-B to sIg^+ B cell have been observed in rabbit and human bone marrow cells (71-73) but it is yet unclear if the latter (sIg^+ B cells) are derived from the former (65,74). These cells from murine neonatal liver or adult bone marrow are immature, early developing sIg^+ B cells and they are extremely sensitive in an irreversible fashion (70) to suppression or elimination by anti-μ (70,75). B cells from adult spleen or lymph nodes require approximately 10-30 times more anti-μ to achieve comparable

TABLE I

SELECTED LIST OF TD, TI-1 AND TI-2 ANTIGENS

ANTIGEN	TYPE	REFERENCE
SIII[1]	TI-?	26,27
SIII-RBC	TD	26,27
TNP-*Brucella abortus*	TI-1	51
TNP-lipopolysaccharide	TI-1	55
TNP-Ficoll	TI-2	8,9
TNP-Dextran	TI-2	7.56
TNP-KLH	TD	57
FLU-polymerized flagellin	TI-?	37
TNP-T_4 bacteriophage	TI-?	58

[1] Abbreviations used: SIII, pneumococcal polysaccharide type III; TNP, trinitrophenyl; FLU, fluorescein.

suppression which in this case is reversible (70). These findings have recently been extended to B cell development in the chicken (76). Similarly, neonatal B cells are more susceptible than adult B cells to anti-μ blockage of the B lymphocyte maturation to plasma cells induced by LPS (61). Furthermore the mechanisms involved in suppressing neonatal and adult B cell maturation by this method appear to be distinct (77) in that neonatal cells are suppressed by brief exposure to anti-μ in the absence of LPS, whereas adult cell suppression requires simultaneous presence of both anti-μ and LPS. It is interesting to note that this ability to suppress mice with anti-μ closely parallels the susceptibility of neonatal mice to antigen-induced tolerance (61,70,75,78-80).

As B cells mature, the density of sIgM decreases (81) and the appearance of another sIg molecule, IgD, is observed (82). The need for two surface isotypes is unclear, although Vitetta and Uhr (82) have hypothesized a correlation between acquisition of sIgD and loss of susceptibility to tolerance. This seems less likely in light of the recent findings of Kearney et al. (83) who have shown that although sIgD appears by day 3 after birth adult levels are not reached until almost 3 weeks of age. The presence of Ia antigens on B cell surfaces more closely corresponds to loss of tolerance susceptibility (83, see below). Most splenic B cells in mice over three weeks of age bear both sIgM and sIgD (83) and a very small percentage of mature B cells express sIgG in addition to IgM and IgD and some express IgG alone or with IgM only (84). On an individual cell both isotypes have identical specificity for antigen (85-87) based on idiotypic specificity (85), antigen binding (86) and co-capping with antigen (87); thus it appears that all the sIg molecules on a single cell share the same V_H gene products regardless of

the C_H specificities displayed. Although this has not been confirmed by sequence data it appears likely since lymphocytes cloned by limiting dilution and adoptive transfer can give rise to progeny synthesizing different Ig classes but maintaining the idiotype and antibody specificity of the parent cell (88). Also studies of biclonal myeloma proteins (84,89,90) in humans suggest that V_H regions can be shared by different C_H gene products. The V_H regions were shown to be identical on the basis of immunologic, biophysical and biochemical studies including partial amino acid sequences (84,89-91).

The role of sIg in triggering B cells is an area of much debate and will not be dealt with here except as it relates to TI and TD responsiveness. Recently Ligler et al. (92) and Cambier et al. (93) have shown that IgM responses to a TD antigen, TNP-RBC, require the presence of both sIgM and sIgD whereas the response to a TI-1 antigen, TNP-Brucella abortus (TNP-Ba) requires only sIgM. In fact removal of sIgD prior to stimulation with a TD antigen renders the cell susceptible to tolerance (94,95) as was predicted previously by Vitetta and Uhr (82). Thus these authors speculated that sIgM could deliver either a tolerogenic or a triggering signal whereas sIgD receptors could only deliver a triggering one. It should be noted that Zitron et al. (54) have reported differences in anti-δ mediated suppression of primary responses between TI-1 and TI-2 antigens, the former being less susceptible to suppression after anti-δ treatment (54). Also Scott et al. (37) have described tolerance to fluorescein (FLU)-POL another TI antigen but of unknown TI type (1 or 2). However the possibility that FLU-POL might have a TD component was not eliminated (37) as mentioned above (see Section IIA). Thus, it appears as though TD responding B cells have

both sIgM and sIgD and removal of the latter can induce tolerance (37, 94,95) and blockade of the former can prevent triggering (94,95). The picture for TI-1 and TI-2 antigens is less clear but for TI-1 antigens, in contrast to the proposal of Vitetta and Uhr (82), tolerance is not induced by anti-δ treatment nor does anti-δ block responsiveness. It appears as though anti-δ can suppress TI-2 responses (54) hence TI-2 responding cells are $sIgM^+$ and $sIgD^+$, however no data on tolerance are available.

2. Fc Receptor (FcR)

FcR is found on fetal liver cells as early as day 12 of gestational age about the same time or perhaps slightly prior to sIg (96). The functional significance of FcR and B cells will require further investigation. Although no studies have been reported correlating FcR with TD and TI responses, it appears an unlikely prospect because of its early ontogenetic development and because it is also present on some T cells, macrophages and polymorphonuclear leucocytes.

3. Gene Products of the I region of the H-2 Complex, I-Associated (Ia).

Murine lymphoid cells which express Ia are predominantly B cells (97-99). By nine days after birth approximately 90% of sIg^+ cells in murine spleen also express Ia (83), and studies by Hammerling et al. (100) and Watson (101) suggest that sIg^+, Ia^- lymphocytes give rise to sIg^+, Ia^+ cells. Accordingly, pre-B cells from adult bone marrow and

neonatal liver do not express Ia determinants and 50-60% of the immature sIg^+ cells in these tissues are Ia^- (83). Just as anti-Ia antiserum can block LPS-induced proliferation (102) and differentiation (100) of B lymphocytes anti-Ia can also be used to inhibit specific antibody responses to antigenic challenge (103). Recently Mond et al. (103) suggested that a quantitative difference could be detected in the expression of Ia on TI-1 and TI-2 responding B cells based on the dose dependent inhibition of an anti-TNP primary response using anti-I region antibody and complement (C); TI-2 cells were inhibited with smaller amounts of anti-Ia and C. This was shown not to be an effect on accessory cells (i.e. macrophages) which also express Ia determinants (104) and whose function would be eliminated by anti-Ia and C (105). Hana Golding (personal communication) has been able to eliminate both TI and TD IgG precursor B cells using anti-Ia and C. However in these experiments no attempt was made to detect possible quantitative differences. Press et al. (106), looking only for the presence or absence of Ia determinants, have found that nearly all primary and seconday B cells responding to TD antigens are Ia^+ and give rise to both IgM and IgG responses. However among primary B cells there is a small population of Ia^- cells which give rise only to IgM. As a result they suggested that Ia might be involved in the M → G switch.

4. Complement Receptor (CR)

The acquisition of CR by B cells occurs after Ia expression (81,100). The rate of CR^+ development varies among mouse strains (81) and is believed at least partially to be under H-2 control (107).

Reports from several laboratories in which various means to deplete complement *in vivo* and *in vitro* were used have suggested a correlation between TD responsiveness and CR^+ lymphocytes (15,42-44, 109). Pepys (42) observed that cobra venom factor (COF)-treated animals markedly depleted of C3 showed impaired immune responses to a thymus-dependent antigen, sheep RBC, but not thymus-independent antibody responses. COF has been identified as a modified C3 which can deplete C3 via factor B of the properdin system (108). Of particular note was the impairment of IgG secondary responses as well as the development of IgG memory. Waldmann and Lachmann (44) reported that a purified IgG anti-mouse C3 reagent inhibited the TD response to TNP-KLH but not the TI response to TNP-LPS. Feldmann and Pepys (109) reported similar findings. However in the experiments of the former investigators a purified $F(ab')_2$ anti-mouse C3 reagent was found to be incapable of producing such inhibition even at 16 times the inhibitory concentration of the intact antibody. Thus it appears as though the observed suppression was not due to C3 consumption by the antiserum but rather a consequence of the C3-anti-C3 complexes generated. Waldmann and Lachmann (44) also reported another cautionary note regarding COF in that it is contaminated with phospholipase A which can give rise to inhibitory fatty acids. Lewis et al. (15) after confirming the *in vivo* depletion of C3 by COF separated CR^+ and CR^- cells by rosetting and reported that CR^- but not CR^+ cells responded to TI antigens. In contrast they also found that although the response to TD antigens was greatly reduced in the CR^- population it was not eliminated completely (43). They proposed that TD and TI antigens stimulate separate subpopulations but that a portion of CR^- cells do cooperate with helper T cells in a TD response but via a

different activation pathway (43). Parish and Chilcott have similarly shown that adoptively transferred CR^- cells respond to SRBC which is TD (110). Although one could argue that in these experiments CR^- cells developed into CR^+ cells prior to responding to the TD antigen, this criticism seems less likely in the experiments of Lewis et al. (43) cited above since anti-C3 was present throughout the culture period and should have been able to prevent C3 interaction even if $CR^- \rightarrow CR^+$. It is of some interest that Hoffman et al. (111) have recently reported that CR^+ and CR^- populations respond to red cell antigens but that the mechanisms for initiation differed. Thus CR^+ cells could cooperate with primed as well as unprimed T cells; macrophages were required but could be replaced by 2-mercaptoethanol (2-ME). On the other hand the CR^- subset could only cooperate with primed T cells, and macrophages although still essential, could not be replaced by 2-ME. Thus it appears that CR is not a useful marker to distinguish the B cell subpopulations which respond to TI and TD antigens. Although it appears that there is CR subset overlap in the response toSRBC it should be noted that all of the above studies considered SRBC to be a totally TD antigen even though the original designation of B1 and B2 as TI and TD responding populations respectively was based on the belief that the SRBC was a mosaic of TI and TD determinants (28).

5. Other B Lymphocyte Antigens

Several investigators have described antisera which can readily distinguish surface antigens on B lymphocytes. The mouse specific B lymphocyte antigen (MBLA) was described by Raff et al. (112,113) who

immunized rabbits with lymph node cells obtained from mice which had been thymectomized, lethally irradiated and reconstituted with syngeneic fetal liver cells. After appropriate absorptions the antiserum was found to be cytotoxic for murine lymphocytes that were negative for the θ antigen i.e. non-T cells. A similarly prepared antiserum has been shown to be cytotoxic for immature B cells as well as plasma cells (114). The antiserum is distinct from anti-Pca 1.1 (see below) in that its cytotoxicity for myeloma cells is completely removed by absorption with bone marrow cells. Anti-plasma cell antigen (Pca 1.1) antiserum was described by Takahashi et al. (115) who immunized DBA/2 mice with histocompatible BAlb/c myeloma tumor cells and found the resulting antiserum to be cytotoxic for myeloma cells. Subsequently the antigen, Pca 1.1, has been shown to be present on normal plasma cells after stimulation with antigen but not on unstimulated precursor cells (116). Thus as mentioned above Pca 1.1 is distinct from MBLA. However like MBLA, Pca 1.1 is not found on T cells (112,113,117). This point distinguishes Pca 1.1 from the activated lymphocyte antigen (Ala-1) of Feeney and Hämmerling (118). The Ala-1 antigen is only found on activated lymphocytes but it is shared by activated T cells and B cells, and like Pca 1.1 is found on mitogen stimulated cells (118) as well as antigen stimulated cells (119). Kakiuchi et al. (120) have described another anti-B cell heteroantiserum prepared by immunizing rabbits with lymph node cells from athymic nude mice. Although similar to MBLA antiserum in that presumably non-T lymphoid cells were used to immunize, this antiserum is distinct from anti-MBLA as well as anti-Pca 1.1 in that it does not kill myeloma cells or antigen-induced plasma cells; thus it appears to recognize a differentiation antigen present on antibody forming precursor cells but absent

or in low concentration on plasma cells.

Since none of the above antisera have been tested for their ability to distinquish between B cells responding to TD and TI antigens, it is not possible to predict whether a differential effect on precursors or blasts of TI and TD responding B cells might be observed. There are however three antisera for which such distinctions have been tested. Gorczynski has prepared several anti-mouse B cell antisera by immunizing mice with B cells from various tissue sources (121). One of these antisera (anti-bone marrow stem cell) could distinguish between TD and TI responding primary B cells from spleens but not bone marrow (122). Huber et al. (123) have reported the preparation of an antiserum in CBA/N F_1 mice which, as mentioned above, carry an X-linked B cell defect and do not respond to TI-2 antigens (50,51,53). They immunized CBA/N F_1 male mice which have the defect with normal parental spleen cells and obtained an antiserum which stained approximately 50% of B cells in an immunofluorescence test. The antiserum, anti-Lyb3, does not stain T cells or macrophages and optimal staining of B cells is not achieved until mice are approximately 2 months old. Unfortunately this antiserum is non-cytotoxic. Finally Ahmed et al. (124) using similar reasoning prepared anti-DBA/2 spleen cell antibody in C57Bl/6 mice and then extensively absorbed the antiserum with tissues from (CBA/N x DBA/2) F_1 male defective mice. This antiserum, anti-Lyb5, was specifically cytotoxic for B cells and like the anti-Lyb3 antiserum, reacted with an antigen which appears late in ontogeny. Although it is not likely that these antisera recognize a single unique determinant (anti-Lyb5 is apparently contaminated with a second specificity, anti-Lyb7 - Ahmed personal communication), they can distinguish at least in

part between B1 and B2 subpopulations. It would be of extreme value to have monoclonal antibodies produced using the powerful tool of hybrid cell technology (125).

6. Physical Differences

In addition to the various surface markers used to describe B cell development above, B cell populations at various stages of maturity have been distinguished by physical means. Such physical parameters as density, sedimentation velocity (a function of size), electrophoretic mobility and glass adherence properties have been instrumental in revealing the complexity of B cell development. Lafleur et al. (126) described an early B cell distinguished by sedimentation velocity (size) which is sIg^+ as assayed by cytotoxic procedures and which gives rise to virgin (i.e. antigen inexperienced) B cells in 3-7 days after isolation and adoptive transfer (127). Unfortunately they term this cell a pre-B cell which is in contradistinction to the fetal pre-B cell mentioned above which is sIg^-. Shortman et al. (128) studying B cells specific for the hapten 4-hydroxy-3-iodo-5-nitrophenyl, NIP, have reported a B cell stage which closely resembles the pre-B cell described by Lafleur et al. This cell, recently termed pre-progenitor (129), is a small early developing B cell which has a distinct density profile reminiscent of a dense memory B cell (130). Pre-progenitor B cells have been shown to accumulate in neonatal germfree mice (130) and are found in elevated numbers in neonatal specific pathogen free (SPF) mice (131). This cell population can be transformed into the medium-sized, medium-density forms characteristic of conventional adult mouse splenic B cells

by exposure to environmental stimuli (130) or by experimental stimulation of the germfree neonate with unrelated antigens (132) twenty-four hours prior to density separation of spleen cells. Adult splenic B cells from conventional mice can undergo a similar non-specific activation to a less dense cell type. The non-specific activation presumably requires adult macrophages since spleen cells from SPF neonates can only shift to a medium density cell type (i.e. like non-stimulated conventional adult mouse cells) in the absence of adult macrophages or in the presence of additional neonatal macrophages; but when adult macrophages are added prior to non-specific stimulation the SPF neonatal cells can make the second shift in density like adult cells. Although these density shifts are induced by so-called non-antigen specific stimuli such as POL alone or horse RBC, similar results are obtained if the mitogen PPD is used (47, 131). A slight density shift is found in a memory cell population forty-eight hours after a non-specific stimulation (131) suggesting a similar pre-progenitor population for memory B cells as well (129). One should be aware that non-specific activation steps have been suggested by a number of groups. Lafleur et al. (126,127,133) and Miller et al. (134) have found a large Ig^+ cell in spleen and bone marrow which matures into a smaller, immunocompetent B cell. Similarly Strober (135,136) has postulated a sequence of development from small dense lymphocytes to larger, less dense cells to small dense cells. Although these authors observed these changes as spontaneous, it could actually be the result of non-specific activation by environmental stimuli. Thus on the basis of density at least two perhaps three distinct virgin B cell populations can be identified: 1) that resembling a B cell found in neonatal germfree animals and I might add present,

but in low numbers, in conventional mice (130,131), 2) that resembling the B cell found in conventional adult mouse spleens and 3) that found in non-specifically activated conventional mouse spleens. In addition there could be at least two density populations of antigen-experienced memory cells, however the data for these are less convincing.

Memory B cells differ from virgin B cells in that the former are classically very dense cells which recirculate to the lymph (135,137). It should be noted that although thoracic duct cells from primed mice have been confirmed as typical dense memory cells (128,138), a population of memory cells in spleens have been found to be moderately dense to dense (128, 138). These cells could be non-specifically activated or newly developing memory cells. These cells are very similar to virgin B cells in density (128). In addition to density differences which can be observed between virgin and memory B cells, these cells can be distinguished by other physical means. Memory cells have been shown to be related to slower migrating populations of cells by electrophoresis (139). These slowly migrating cells are more typically Ig^+ cells whereas the faster migrating cells are Θ positive (140,141). These separation data should be viewed with caution however since the separated populations were not depleted of T cells prior to adoptive transfer. Thus possible suppression of memory B cell expression in the predominantly T-cell region cannot be excluded. This is a particular problem in Shortman's system since their assay for virgin B cell responses uses a TI antigen and their assay for memory B cell responses used a TD antigen (141,143). This is most unfortunate in that the differences observed might be more related to their use of two different antigenic forms than to virgin versus memory B cells since at least partial

separation of TI and TD responding virgin cells has been achieved by velocity sedimentation (31).

Conflicting results have been reported for virgin B cell sedimentation velocity profiles. Fidler et al. (130) and Shortman et al. (128) reported NIP progenitor B cells to be large, fast sedimenting cells, atypical of the majority of sIg^+ cells. Others report virgin progenitor cells to be smaller, slowly sedimenting typical B cells (126,127, 133,134). In all the latter studies a TD antigen (SRBC) was used versus a TI antigen (NIP-POL) in the former studies. Could this explain the differences? Again as mentioned above the possibility has been suggested by the data of Gorczynski and Feldmann (31) who could enrich for TI (rapidly sedimenting, larger cells) or TD (smaller and slower sedimenting cells) responding cells on the basis of sedimentation at unit gravity. Memory cells appear to sediment as typical B cells (128,138).

Finally, partial separation of IgM and IgG progenitors has been achieved by passage of cell suspensions in serus containing medium through columns of large, siliconized glass beads (144,145). Under defined conditions several groups have reported differential adherence between IgM and IgG progenitor cells. Shortman et al. (146) described adherent cells as low density cells characteristic of virgin progenitors whereas the non-adherent cells were of high density. In addition adherent cells contain most of the electrophoretically fast migrating B cells (141). These data are in agreement with those of Schlegel and Shortman (144) and Schrader and Vadas (145) who have also reported that unprimed presumably virgin progenitors are the adherent cells and that memory progenitors are relatively non-adherent.

Although much work has been done on physical separation of cells

only one paper has attempted to study TI and TD responding B cell subpopulations (31) by this means. It is clearly important to use these separation techniques in studies comparing TD and TI antigens as well as the other surface markers described above. Discrepancies in the literature on virgin IgM B cell sedimentation velocity could be explained on the basis of different molecular forms of antigen being used. Alternatively the differences could be due to one group using a single haptenic group, NIP, as antigenic determinant and the other using a multideterminant one, SRBC If the former proves correct it may provide the basis for the isolation or enrichment of the individual populations.

C. Ontogeny of Responsiveness to TD and TI Antigens.

Unresponsiveness of neonatal animals to specific antigenic challenge, notably TD antigens, has been widely reported (147-154). Since these same neonatal animals could respond to TI antigens (154) and/or B cell mitogens (148) it has been assumed that TI responsive B cells must develop prior to TD responsive B cells (34,155). However the failure of neonates to respond has since been attributed to lack of T cell function (148,156). Some authors have proposed the presence of suppressor T cells. For example this was suggested by Hardy et al. (154) since the TI response in athymic nude mice was higher than the response in their normal siblings. Recently Mosier and Johnson (156) have more carefully studied B cell responsiveness in neonatal mice. Their results confirm this prediction and clearly show that neonatal spleen cells can respond to TD antigens if suppressor T cells are first removed and an adequate source of help added. The suppressive activity was highest

at birth and declined thereafter such that adult responses were achieved by 6 weeks. The response to DNP-ficoll was also suppressed but not to the same extent as TD responses. Thus the response in neonatal mice to TD antigens was not being detected because of suppression while the response to TI antigens was apparent at birth.

Howard and Hale (157) compared the immune response of 14 day old and adult CBA mice with DNP-ficoll (TI) and DNP-KLH (TD). Whereas the 14 day old response to DNP-KLH was approximately 91% of the adult level the response to DNP-ficoll was only 7% of the adult response. Thus the ability of CBA mice to respond to TD antigen antedated the development of the response to a TI form of the same hapten. These data may not be in conflict with those of Mosier and Johnson (156) since a different strain of mouse was used and at 14 days of age suppressor activity was diminishing as studied by Mosier and Johnson. Similarly, although CBA/N mice have an impaired response to TD antigens, it is nevertheless the response to TI-2 antigens which is absent (155). If the CBA/N defect is in a population of mature B cells (158-160) then CBA/N mice would be correspondingly rich in immature cells. Accordingly the CBA/N mouse could agree with the data of Howard and Hale (157) in that TD responsiveness precedes TI responsiveness. However, it should be noted that Kincade has proposed (161) that the CBA/N defect is in an immature population since they totally lack the ability to form B cell colonies in soft agar, a trait found very early in murine neonatal development. Since colony forming cells are inhibitable by anti-μ antibodies (162), they are already relatively mature (sIg^+) and their appearance early in neonatal development may only reflect the different rates at which B cells and the repertoire develop (152, 153, 163, 164). It has been suggested by

numerous authors that development of the B cell repertoire is a highly ordered non-random maturational event (152,153,164) which begins in the mouse during the last week of gestation for some antigens while for others significant levels are only achieved at about 6 weeks of age (147,148).

Finally many investigators have studied the relative susceptibility of neonatal (by definition immature) and adult cells (by definition mature) to tolerance induction. Four systems for tolerance induction and assessment have been used. In the first a TD antigen was used as tolerogen as well as the challenge antigen (165). In these instances neonatal spleen cells (or adult bone marrow cells) were found to be approximately 1000-fold more sensitive to TD induced B cell tolerance than adult spleen cells. In the second case, TD antigen was used as tolerogen but TI antigen was used as the challenge antigen (34). Using this system Cambier et al. (34) reported that unlike the results obtained above (165) no difference in susceptibility to tolerance induction could be detected when tolerant cells were challenged with a TI antigen. Similarly, Siskind et al. (166), Howard and Hale (157) and more recently Tite et al. (167) have studied the response of TI tolerant cells to TI antigens. The results obtained here were similar to those observed previously when TI antigens were used to challenge TD tolerant cells i.e. no difference between neonatal and adult cells in tolerance susceptibility was observed. In addition Tite et al. (167) have also studied TD responses of TI tolerant cells and have found similar results to those obtained with TD tolerant cells, namely TD responding B cells are less susceptible to tolerance induction than are TI responding B cells. Since tolerance can be induced by a variety of mechanisms, e.g. clonal

abortion (168,169), receptor blockage (170) or induction of suppressor T cells (171-173), it is difficult to make valid comparisons from one system to another. However, it appears based on the above data as though in these systems the ability to respond after tolerance induction is related less to the tolerogen used than it is to the differentiative state of the cells being challenged. Thus distinction can clearly be made between B cells responding to TD and TI antigens based on the ease of tolerance induction.

A final word concerning the ontogeny of B_1 and B_2 cells is warrented. Three hypotheses for the ontogenetic development of B_1 and B_2 have been proposed. Cambier et al. (34) suggested a common pathway of development from stem cell $\rightarrow\rightarrow$ $B_{1\mu} \rightarrow B_{2\mu}$ $\rightarrow$ memory based on properties which B_1 cells in the adult have in common with immature B cells i.e. ease of tolerance. This view has fallen into disfavor in light of the above data on development of TI and TD responsiveness and because of the CBA/N mouse. An alternative view is that $B_{2\mu}$ cells may be the precursors of $B_{1\mu}$ cells. This was originally proposed by Andersson and Blomgren (174) who observed that although polyvinyl pyrolidone (PVP) was TI when tested in adoptive transfer using adult spleen cells, it was TD when transferred with spleen cells from two week old mice. In fact, the PVP response of adult spleen cells was suppressed when T cells were transferred simultaneously. Thus they hypothesized that immature B cells required T cell help whereas mature B cells did not require T cells but could be regulated by their presence. Quintans and Cosenza (33) also favor $B_2 \rightarrow B_1$ since they sometimes observed synergistic (i.e. greater than simple addition) responses to phosphorylcholine (PC) but only when the cells were challenged first with the TD form and then with the TI

form 24 hours later. Finally Mosier et al. (50) have used $B_2 \rightarrow B_1$ as a possible explanation for the CBA/N anomaly (50,51,53). The third possible ontogenetic scheme is that B_1 and B_2 are separate lineages of cells as suggested by Kincade (161, see above) who found CBA/N mice to be devoid of colony-forming B cells (immature, B_1?). The data presented to date are compatible with either of the latter two models for B_1 and B_2 ontogeny. In light of the objection to the model proposed by Kincade I tend to favor the $B_2 \rightarrow B_1$ scheme although no conclusive evidence is available.

Furthermore, it should be emphasized that attempts to correlate tolerance of TI or TD responses with tolerance of neonatal or mature cells may not be as straight forward as expected since one does not know the role(s) of accessory cells in overcoming the tolerant state.

D. Evidence for TI and TD Subpopulations of Primary B Cells (B_1 and B_2).

In addition to the above reports of differential requirements for C3 and differential sensitivity to tolerance induction by TD and TI antigens there have been several studies of humoral immune responses which also indicate that separate subpopulations respond to each type of antigen (28-33). Playfair and Purves (28) suggested on the basis of adoptively transferred cells in limiting dilution assays that synergy between thymus and spleen or bone marrow in the anti-SRBC responses was not proportional to the reactivity of either spleen or bone marrow cells in the absence of T cells. Based on these results they proposed two subpopulations of B cells one of which B_1 did not require T cell help to generate an immune response whereas the second subpopulation, B_2 did.

This concept was supported by velocity sedimentation analysis of primed and unprimed B cells prior to in vitro challenge with TI or TD hapten-carrier conjugates (31). Two populations of responding cells could be distinguished in spleen and bone marrow (but not in lymph node), a population of small cells responding to TD antigens and a population of rapidly sedimenting larger cells responding to TI antigens. The differences were distinct among IgM precursors but subpopulations could not be distinguished among IgG precursors (this could reflect the fact that IgG responses were much lower being only 25 - 40% of the comparable maximum IgM responses). Recently Jennings and Rittenberg (32) reported that when both TD and TI forms of the TNP-hapten were added to the same spleen cell cultures, the response to the hapten was additive. Additive responses only occurred if the optimal concentration of each antigen was used. If a suppressive dose of one antigen was used to double challenge a culture along with an optimal dose of the second, the anti hapten response to the second antigen was not impaired. Addition by double antigen challenge only occurred when combinations of TI and TD antigen were used; addition did not occur when two TD or two TI antigens were added to the same culture. Similar in vitro additive responses between TD and TI forms of PC were obtained by Quintans and Cosenza (33) who used limiting dilution analysis and found that the number of TI precursor B cells was 3-10 times greater than the number of TD precursors. They also noted that the burst size of PFC from individual precursors stimulated by the TD antigen was 4-5 times greater than that stimulated by the TI antigen. They concluded that TD and TI antigens stimulate different B cell subpopulations. A similar conclusion was drawn on the basis of small differences in the kinetics of development of IgG memory

expression *in vivo* to TD and TI forms of SIII (26). In addition Lewis et al. (15,43) separated CR^+ and CR^- cells by rosetting techniques. As mentioned above they found that the CR^- but not CR^+ cells responded to TI antigens. Although the CR^- population had a partial TD response the CR^+ cells were enriched for TD responding B cells.

E. Evidence Presented Here.

In this thesis I shall present data related to B_1 and B_2 obtained using a new model system for studying resting memory B cells *in vitro* (see MSI). This system allows the expression of both TD and TI IgG memory responses after priming with the TD antigen form. Thus the *in vitro* system resembles the *in vivo* system of Braley-Mullen (14,26, 27) and the adoptive transfer system of Schlegel (142,143). It is necessary to point out that Schlegel claims that his secondary response is totally T dependent. Although an enhanced response is seen when he uses helper T cells, he nevertheless has observed a distinct TI IgG response after TD priming whereas the primary TI response is totally IgM (142); however he appears not to recognize this aspect of his results.

The data shown in MSII-IV present a clear picture in testament of subpopulations of hapten specific memory B cells. The memory responses to TD and TI forms of the same hapten are additive both at the plaque-forming cell level and at the precursor level (MSII and III) as has been shown for primary responses. Secondly, since memory cells require several proliferative cycles in order to develop into antibody secreting cells, (175,176) they should be more susceptible to treatment with cytotoxic drugs which act on dividing cells than are primary B cell subpopulations. The data presented indicate that unlike primary B cell

subpopulations which have not been distinguished by killing in this method (33), G. Lewis personal communication) the secondary IgG subpopulations can be eliminated selectively with bromouridinedeoxyribose and light providing the strongest evidence for subpopulations of B cells (M SIII and IV). Lastly, evidence is presented which may form the basis for distinguishing between TI-1 and TI-2 type antigens in that the former appear to stimulate both B_2 and B_1 cells in a T independent manner whereas the latter only stimulate B_1 cells. Thus the ability to stimulate B_1 and/or B_2 cells cannot be exactly correlated with the relative thymus dependence of the immunogen. It remains to be determined what properties are associated with TI-1 and TI-2 antigens, but for the present TI and TD are useful terms.

Manuscript 1

Expression of IgG Memory *In Vitro* to Thymus Dependent and Thymus Independent Antigens

ABSTRACT

A model is described in which expression of IgG secondary anti-hapten responses of large magnitude can be initiated *in vitro* without resorting to *in vivo* boosting prior to culture. The number of IgG PFC is frequently as much as 100-fold greater than that of IgM PFC. Spleen cells from mice primed with trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) several months earlier are stimulated *in vitro* to produce an anti-TNP plaque-forming cell (PFC) response 7-10 days later. The *in vitro* IgG response can be elicited with either a thymus-dependent antigen (TNP-KLH) or thymus-independent antigens (TNP-T_4 bacteriophage or DNP-dextran). The kinetics of the responses to these two forms of antigen differ in that the thymus independent response peaks two days earlier. The IgG response to both forms of antigen requires the presence of 2-mercaptoethanol (2-ME) even though macrophages are not depleted prior to culture. In the absence of the reducing agent both thymus dependent and thymus independent IgG responses were diminished $\geq$ 90%. The magnitude of the response to thymus independent antigens emphasizes the ability of these materials to elicit IgG expression in memory B cells provided optimal conditions for memory development and *in vitro* expression exist.

INTRODUCTION

Adoptively-transferred primed spleen cells give normal secondary anti-hapten responses in recipient mice (177-179) but only rarely do so *in vitro*; usually IgG PFC are deficient *in vitro* (177,180-182). The inability to generate anamnestic responses *in vitro* comparable to those obtained *in vivo* has made it difficult to study many of the questions related to the regulation of expression of IgG production in a controlled environment. Previous reports from this laboratory and others (2,177, 182) have discussed this difficulty, and it has been shown that it could be overcome by giving primed mice an *in vivo* boost of antigen prior to culturing *in vitro* (177,182,183). However, the necessity for boosting makes analysis of resting memory cell populations impossible since the B cells giving rise to the response in this type of system are probably not resting memory cells but rather cells which have already been activated (177) and have begun the maturation sequence required for IgG secretion (175,176) and which may involve several replicative cycles (175). Thus it seemed that if resting memory B cells were to be studied, primed spleen cells had to be cultured without prior boosting and for a sufficient duration to allow for a multiplicity of replicative cycle.

We have found that TNP-primed mouse spleen cells can give rise to an anti-TNP IgG response if cultured for 7-10 days (184). Here we describe the kinetics of the *in vitro* memory response to thymus-dependent TNP-KLH (38) and thymus-independent TNP-T4 bacteriophage (58) or DNP-dextran (56) forms of the hapten. The response requires the presence

of 2-ME. The magnitude of the response and the relative values for IgG and IgM are comparable to those reported previously for *in vivo* secondary responses or for *in vitro* responses in which the spleen cell donors had been boosted prior to culture (2,177-182).

MATERIALS AND METHODS

Animals. Adult Balb/c female mice were purchased from Simonsen Laboratories, Inc., Gilroy, Calif. They were caged in groups of six with free access to food and water and were used when two to eight months of age.

Antigens. Trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) was prepared as described previously (57) and had a mole ratio of $TNP_{1067}KLH$. Particulate TNP-KLH was prepared by coating TNP-KLH onto bentonite (T-K-B) as described previously (185). TNP-T4 bacteriophage and $DNP_{10.5}$ dextran were gifts from Dr. John Jennings and were prepared as described previously (56,58).

Immunizations. Mice were given intraperitoneal injections of T-K-B once a week for three weeks. Each injection contained 100 µg protein in 0.5 ml saline.

Cell culture. Spleen cells from at least 3 mice were cultured using microtiter plates (186) which reduced all volumes to 0.1 of those used previously (180). Culture medium was supplemented with 5×10^{-5} M 2-ME (186), unless otherwise stated. Each microculture contained 10^6 cells/well and the contents of 8 such wells were pooled for PFC

assay as one culture. Three such cultures were assayed per experimental point to obtain data for statistical analysis. More recently we have supplemented the culture medium with nucleosides according to Click et al. (187). These cultures were fed with 1/2 volume of nutrient cocktail and nucleosides on day 1 only and they were not rocked. The results obtained using this technique were equivalent to those obtained in the standard micro Mishell-Dutton.

Detection of in vitro anti-TNP response. TNP-haptenated sheep red blood cells (TNP-SRBC) were prepared according to the method of Rittenberg and Pratt (185). Cells secreting anti-TNP antibody were detected by plaque assay using TNP-SRBC and the slide technique of Cunningham and Szenberg (188). Cells producing IgM anti-TNP antibody were detected by direct plaquing with guinea pig serum (1:3) as a source of complement. Cells producing IgG anti-TNP antibody were detected by adding goat antimouse IgG antiserum and complement. The latter assay was conducted in the presence of sheep anti-MOPC 104E (IgM, λ) antiserum which suppressed IgM plaques by $\geq$ 95% (189). The anti-MOPC 104E was kindly provided by Dr. A. Malley.

RESULTS

Dose response to a thymus-dependent antigen. Previously we showed that TNP-KLH initiates a vigorous secondary antihapten response in vitro which peaked on day 5 in conventional Mishell-Dutton cultures (180). Figure 1a shows the results of a typical dose response experiment in microculture in which spleen cells from T-K-B primed mice were assayed

after 5 days of culture in the presence of various doses of TNP-KLH. The spleens were obtained from mice 29 weeks after priming, but similar results were obtained in seven other experiments using mice 12 to 30 weeks after priming. As reported previously concentrations of antigen greater than those yielding maximum stimulation suppressed the response (180). Lower doses than those shown here did not give as high an IgM or IgG response although the IgG/IgM ratios were maintained and occassionally were higher.

Mitogen studies have suggested that IgG precursors may undergo several proliferative cycles prior to secretion (175). Consequently, replicates of the above cultues were maintained in tissue culture an additional 4 days and assayed on day 9 for PFC. As can be seen in Figure 1b, there was a large increase in the IgG response; whereas the IgM response remained virtually unchanged. As a consequence the IgG/IgM ratios on day 9 are much higher than those for day 5 with 3 of the 5 doses yielding ratios > 10:1. Both the magnitude of the IgG response and the IgG/IgM ratio are in accordance with _in vivo_ secondary responses to TNP-KLH reported previously (182).

Dose response to a thymus-independent antigen. Recently, it was reported that mice primed with a thymus-dependent antigen can show an anamnestic response (IgG and IgM) to an _in vivo_ challenge with the thymus-independent form of the same immunogen (pneumococcal polysaccharide (26)). Here we tested the ability of T-K-B primed spleen cells to respond to _in vitro_ challenge with TNP-T4 since unprimed spleen cells only give an IgM response to this antigen (58). Cells were cultured

with various doses of TNP-T4 and assayed for anti-TNP PFC on days 5 and 7 of culture. Typical results are shown in Figures 2a and 2b and indicate a strong IgG anti-TNP response to TNP-T4 which, like the response to TNP-KLH shows a high IgG/IgM ratio (10:1 on day 7). The highest dose used here is similar to the optimum dose required for a preliminary in vitro response (58).

Kinetics of the IgG memory antihapten response in vitro. Spleen cells from T-K-B primed mice were cultured with various doses of TNP-KLH or with the optimum dose of TNP-T4 24-29 weeks after priming. The number of cells secreting IgG anti-TNP antibody was assessed at different times as indicated for TNP-KLH in Figure 3 which shows the results from one of eight experiments in which similar dose effects were observed. The data are shown as the mean indirect PFC/10^6 recovered cells $\pm$ standard error. The maximum PFC for all doses was observed on day 9 regardless of kinetics. However, the kinetics are greatly affected by the dose of TNP-KLH 1) low doses have a tendency to cause a more rapid IgG response and higher dose delay the onset of the response; 2) while low doses increase the rate at which the peak response is achieved, they do not alter its magnitude. The response to 0.002 µg/ml TNP-KLH achieved 42% and 84% of the day 9 response on days 5 and 7, respectively; whereas the 0.02 µg/ml dose attained only 53% of the maximum response by day 7. The 0.2 µg/ml dose further delayed the onset of the response achieving only 13% of maximum by day 7. As shown in Figure 1b for day 9, higher doses of antigen completely eliminate the secondary response. Figure 4 compares the responses to an immunogenic dose (0.02 µg/ml) and a suppressive dose (2.0 µg/ml) of TNP-KLH through day 10. Unlike lower

but immunogenic doses of TNP-KLH which only delayed the onset of the response (0.2 μg Figure 3) there was no recovery of responses paralyzed by doses $\geq$ 2.0 μg of TNP-KLH even when cultured beyond day 10 (not shown). In one of eight experiments the decline of the response to immunogenic doses leveled off after day 10, but in the others the response showed a steady decrease until by day 15 it was no different from background.

Kinetics of the response to an immunogenic dose of TNP-T4 are shown in Figure 5 (one of 4 experiments). Although the response to the thymus-independent form of the hapten consistently peaked on day 7 two days earlier than to TNP-KLH, the rate of development is strikingly similar to that of cultures stimulated with 0.002 μg/ml TNP-KLH (Figure 3). However, unlike the response to a low dose of TNP-KLH which could achieve 84% of maximum on day 7 and 60% on day 10, the response to TNP-T4 which reaches maximum on day 7 declines more rapidly (25% of maximum by day 10).

We also tested the ability of DNP-dextran to stimulate T-K-B primed spleen cells to determine if the difference in kinetics of the responses to TNP-KLH and TNP-T4 might be associated with thymus dependency. Spleen cells from mice primed 12 weeks previously were cultured with 10^{-3} μg DNP-dextran and the number of cells secreting anti-TNP antibody was determined on days 5, 7 and 9 of culture. Typical results (1 of 3 experiments) are shown in Table I. DNP-dextran was able to elicit a strong IgG response which peaked on day 7 as does the TNP-T4 response. The IgG/IgM ratio on that day was 23. In addition, like the response to TNP-T4, there is a rapid decline of the response such that by day 9

the response is approximately 8% of that observed on day 7. We have not been able to alter the kinetics of the response to either thymus independent antigen as was possible with TNP-KLH (Figure 3) although high doses of these antigens are suppressive (data not shown).

Similarly, we have tested other thymus independent antigens (DNP-polymerized flagellin (DNP-POL), DNP-pneumococcal polysaccharide-SIII (DNP-SIII), DNP-ficoll, TNP-polyacrylamide) on day 7 of culture, and they also stimulated IgG responses in T-K-B primed cells (not shown) although the kinetics of these responses have not yet been assessed.

2-ME dependence of TNP-T4 and TNP-KLH memory responses. The relative dependence of both IgM and IgG responses to TNP-T4 and TNP-KLH on the presence of 2-ME was studied. Whole spleen preparations from T-K-B primed mice 22 weeks after priming were placed in culture with either TNP-KLH or TNP-T4 in the presence or absence of 2-ME. Macrophages were not depleted in these experiments. The cultures were assayed for anti-TNP PFC on days 5, 7, 9 and 11 of culture. The data shown in Table II clearly indicate two points which we have observed in 3 similar experiments. First, the IgG response to both TNP-T4 and TNP-KLH requires the presence of 2-ME. Secondly, the IgG response to TNP-T4 shows partial recovery by day 11 (35% of control) while that to TNP-KLH is still 90% suppressed. The IgM responses were depressed to a lesser degree than the IgG responses (only 64% for cultures challenged with TNP-KLH and assayed on day 9, data not shown). Additional cultures without 2-ME were challenged with doses of TNP-KLH ranging from 0.0002 μg/ml - 2.0 μg/ml and showed similar results indicating that the dependence on 2-ME is independent of the antigen dose.

DISCUSSION

Previous evidence has suggested that all cells required for a memory response are present in the spleen (177-180) even though IgG expression was limited (177,180-182). The data presented here are in agreement with these observations and suggest that one problem of studying generation of secondary IgG PFC responses *in vitro* has been failure to maintain cultures for a sufficient length of time. The time is presumably needed for a maturational sequence required to direct memory cells into IgG secreting cells (175,176). Furthermore, in experiments not shown, the time requirement could not be replaced by altered serum concentrations or feeding schedules. Independent of our work, North and Maizels (190) recently obtained similar results using a thymus dependent antigen. They were able to obtain such IgG responses in micro Marbrook (191) but not in conventional Mishell-Dutton (192) cultures suggesting that our use of micro Mishell-Dutton cultures may be important; while we have not tested this point in detail we noted previously that T-K-B primed spleen cells responded poorly on day 7 in secondary *in vitro* responses using the conventional culture technique (193).

As shown previously memory cells are very susceptible to high dose tolerance (180,194,195) and Figure 4). The nature of this tolerance is unknown. Receptor blockade has been suggested as one explanation in which avidity maturation of B cell receptors would account for those situations in which memory cells are more sensitive to high dose tolerance than primary cells (180). The kinetics are very different for 0.002

and 0.02 µg TNP-KLH compared to 0.2 µg (See Figure 3). It appears that with the highest dose which is still immunogenic, 0.2 µg, the response is able to mature up to a point short of secretion and that cells may develop secretory ability all at once i.e. final maturation from 15% to 100% of the response occurs in just 24 hours. This suggests that there may be a single reversible event which prevents these cells from expressing and that cells accumulate at the point of block until the inhibitory phase is reversed thus leading to apparent synchronization of the response. While this explanation would fit the receptor blockade hypothesis, we cannot exclude the possibility that short lived suppression develops in these cultures. Both Kontiainen and Feldman (196) and Eardley and Gershon (197) have reported the generation of suppressor Tcells from normal spleen cells in 4 days of culture and this could account for the unresponsiveness observed here with both the reversible dose (0.2 µg) and the irreversible dose (2.0 µg) of TNP-KLH.

An important feature of this model is that it mimics an in vivo situation (26) in that we can obtain memory IgG responses to a thymus independent form of the hapten, TNP-T4 (Figures 2 and 5) or DNP-dextran (Table I). DNP-ficoll, DNP-polyacrylamide beads, DNP-SIII, DNP-polymerized flagellin all stimulate secondary IgG PFC in this system (data not shown). This is in contradiction to results in which in vitro challenge of primed mouse spleen cells with DNP-POL (thymus independent) only gave rise to IgG PFC if a nonspecific T cell supernatant factor was added (23). Thus, it is possible that the thymus independent antigens used here were also activating T cells nonspecifically. However, in the previous study IgG

responses achieved were minimal, and it is possible that either the priming or culture regimen used here allowed for greater memory development and/or expression.

The rate of development of the response to TNP-T4 and DNP-dextran resembles that to the lowest immunogenic dose of TNP-KLH (0.002 g), but the thymus independent responses decline at a faster rate. Whether this is a reflection of the nature of the antigens or of the responding cells is not known. It has been proposed that these forms of antigen stimulate distinct subpopulations in primary responses (15,28,31-33). Preliminary evidence utilizing BUdR and light to eliminate one responding population suggests that this may be true also for IgG memory cell precursors (T.V. Tittle and M.B. Rittenberg, unpublished).

If this view is correct, the IgG subpopulations may share a common lineage since both would have been generated by TNP-KLH priming. Since memory B cells have been obtained in the relative absence of T help (23-25) we cannot exclude the possibility that exposure of virgin B_1 thymus independent precursors to TNP-KLH might result in memory B_1 cells directly without sharing lineage with the B_2 thymus dependent population. It should be noted, however, that Braley-Mullen has shown a requirement for T cells in the induction of IgG memory cells with thymus dependent SIII-RBC but not in their subsequent activation with thymus independent SIII (27).

The role which 2-ME plays in development of the <u>in vitro</u> immune response is unclear. It was suggested originally that 2-ME can replace macrophages in the immune response (198). More recent evidence suggests

that 2-ME acts as a T cell mitogen in conjunction with fetal calf serum (199); that it is required for the action of a macrophage-derived-factor involved in the generation of polyclonal killer T cells (200); and that it enhances the activity of LPS-generated small Ig^+ lymphocytes and improves cell viability (201). It also has been reported to be required for the agar-associated mitogenic activity (not LPS or dextran sulfate) necessary to clone B lymphocytes in semisolid agar (202). It is difficult to eliminate macrophages completely, and it is not always clear whether 2-ME replaces macrophages or enhances the activity of residual macrophages particularly since antigens may differ in their degree of dependence on macrophages (203). Here macrophages were not depleted, and the responses to both thymus dependent and thymus independent antigens were depressed in the absence of 2-ME. IgM responses were less affected than were IgG responses, and depression could not be overcome by either raising or lowering the challenge dose of antigen. As with kinetic data the relative degree of recovery of the response in the absence of 2-ME between the TNP-T4 and TNP-KLH responses is compatible with the concept that these antigens may stimulate separate subpopulations. Since macrophages were not depleted, it is unlikely that it reflects the relative dependency of the two antigens on macrophage help.

While secondary IgG responses to thymus independent antigens have been obtained <u>in vitro</u> previously (23,38,204) they have been of modest proportions. Consequently it has appeared that such antigens were generally inefficient at triggering memory B cell populations. The magnitude of the responses obtained here to thymus independent antigens

emphasizes the ability of these compounds to elicit IgG expression. These results suggest that previously observed limitations in such responses were more likely related to factors associated with the priming regimen and/or *in vitro* culture conditions than with characteristics of IgG memory precursors.

TABLE I

Indirect PFC response of T-K-B primed spleen cells stimulated by the thymus independent antigen DNP-dextran.

Days in culture[a]	Anti-TNP PFC/10^6[b]	
	IgM	IgG
5	222 ± 38	247 ± 119
7	424 ± 81	9751 ± 479
9	113 ± 19	1246 ± 91

[a]Spleen cells from T-K-B primed mice were cultured with 10^{-3} µg DNP-dextran and assayed on the days indicated. Mice were used 13 weeks after priming.

[b]The results are expressed as the mean of triplicate cultures ± S.E.

TABLE II

Dependence of *in vitro* secondary antihapten response upon the presence of 2-ME[a]

Ag[b]	2-ME	Day 5	Day 7	Day 9	Day 11
TNP-KLH	+	1427 ± 267	8238 ± 622	8883 ± 665	5018 ± 231
TNP-KLH	-	25 ± 8	107 ± 17	154 ± 47	384 ± 38
% of 2-ME Response		2	1	0.2	8
TNP-T4	+	6459 ± 1215	15306 ± 154	9028 ± 867	6365 ± 837
TNP-T4	-	10 ± 5	1029 ± 163	872 ± 148	2217 ± 348
% of 2-ME Response		0.2	7	10	35

[a] Spleen cells from mice primed with T-K-B were cultured with antigen in the presence or absence of 5×10^{-5} M 2-ME and assayed for anti-TNP PFC on the days indicated. Mice were used 22 weeks after priming.

[b] TNP-KLH = 0.02 μg/ml; TNP-T4 = 6×10^{6} PFU/ml.

[c] The results indicate the mean of triplicate cultures ± S.E.

Figure 1. Dose response of T-K-B primed nonboosted spleen cells to a thymus-dependent antigen, TNP-KLH . Cells 29 weeks after priming were cultured with antigen for 5 days (1a) left, or 9 days (1b) right in microtiter plates then harvested and assayed as described in the text. Vertical bars represent the S.E.M. of three cultures. The response of cells cultured without antigen has been subtracted.

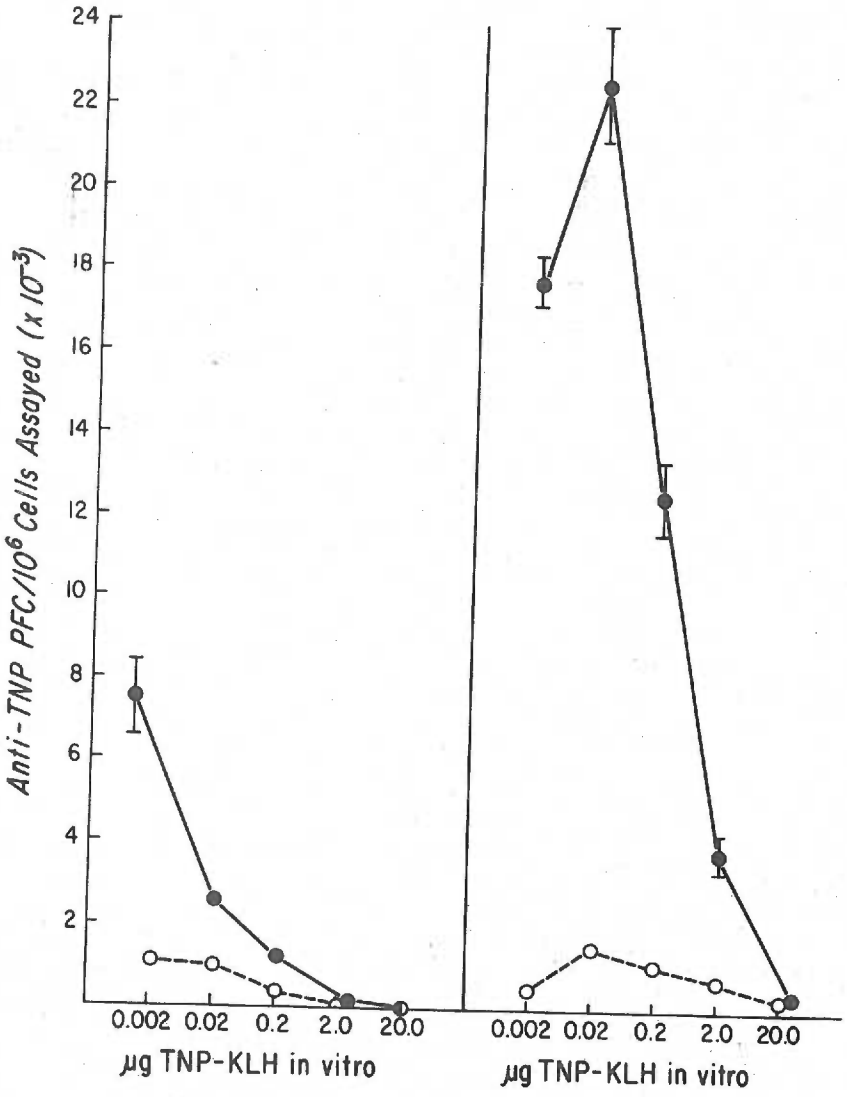

Anti-TNP PFC/10^6 Cells Assayed (x 10^-3)
24
22
20
18
16
14
12
10
8
6
4
2
0.002 0.02 0.2 2.0 20.0
μg TNP-KLH in vitro
0.002 0.02 0.2 2.0 20.0
μg TNP-KLH in vitro

Figure 2. Dose response of T-K-B primed nonboosted spleen cells to a thymus independent antigen, TNP-T4 . Cells were cultured with antigen for 5 days (a) left or 7 days (b) right then harvested and assayed. Mice used 24 weeks after priming. Vertical bars indicate the S.E.M. of three cultures as in Fig. 1.

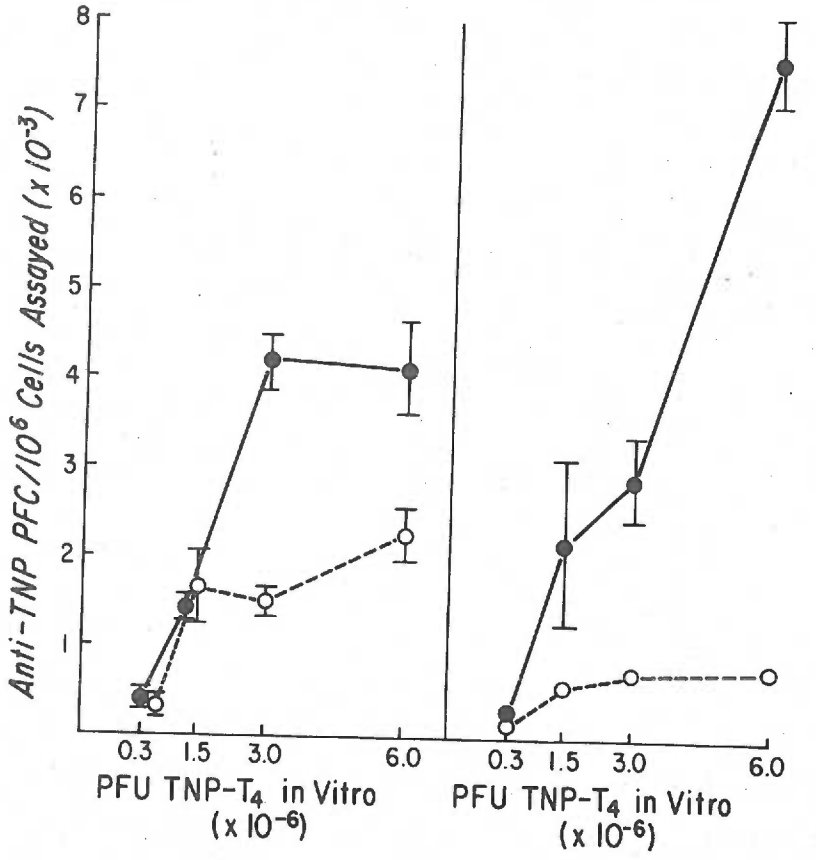
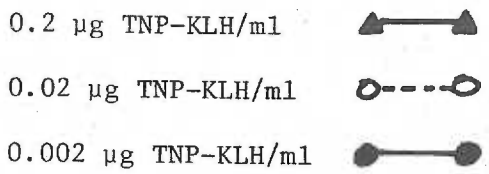

Anti-TNP PFC/10^6 Cells Assayed (x 10^-3)
8
7
6
5
4
3
2
1
0.3
1.5
3.0
6.0
PFU TNP-T4 in Vitro
(x 10^-6)
0.3
1.5
3.0
6.0
PFU TNP-T4 in Vitro
(x 10^-6)

Figure 3. Kinetics of the indirect IgG memory response to TNP-KLH. Primed spleen cells were cultured in the presence of various immunogenic doses of TNP-KLH and assayed on the days indicated. The results are presented as the mean anti-TNP PFC/10^6 cells assayed as a Figure 1 ± S.E.M. Mice used 29 weeks after priming.



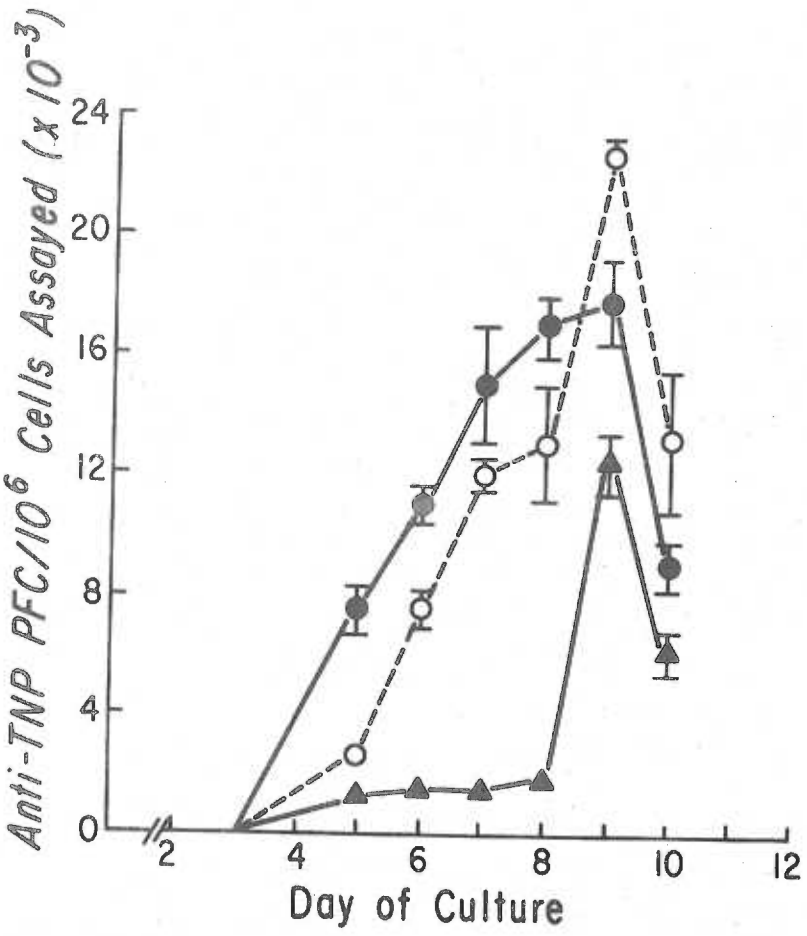

Anti-TNP PFC/10^6 Cells Assayed (x 10^-3)
24
20
16
12
8
4
0
2
4
6
8
10
12
Day of Culture

Figure 4. High antigen dose suppression of the in vitro secondary IgG response. Cells were cultured with either an immunogenic dose of TNP-KLH (0.02 µg/ml, O---O) or a suppressive dose (2.0 µg/ml, ●—●). The data are presented as the mean indirect anti-TNP PFC/10^6 recovered cells as in Figure 1. Mice used 24 weeks after priming.

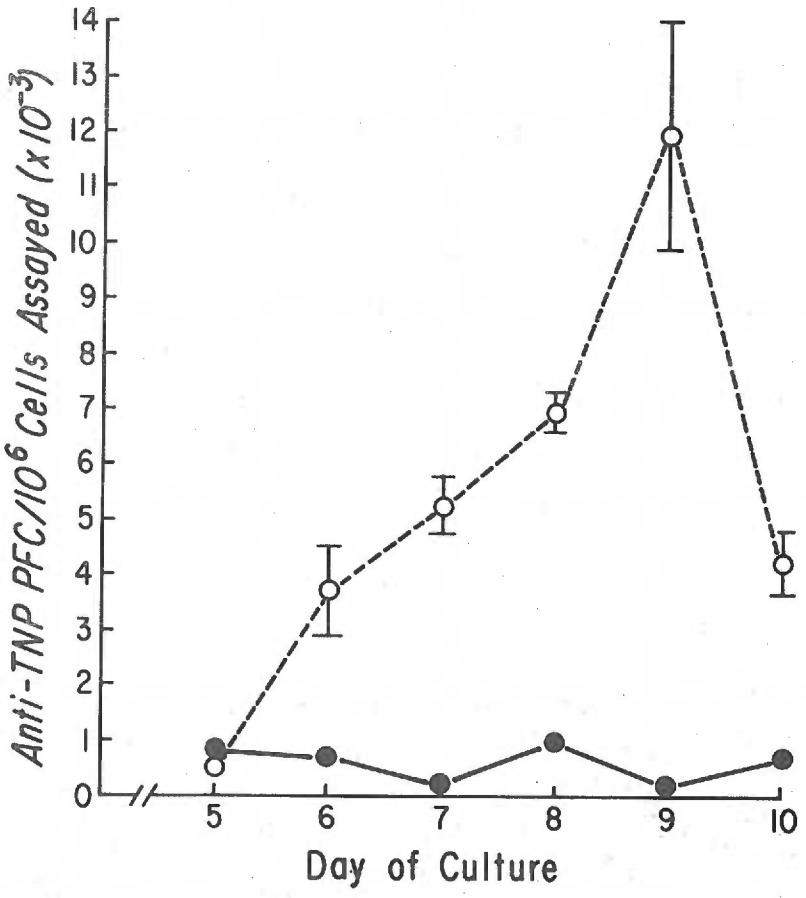
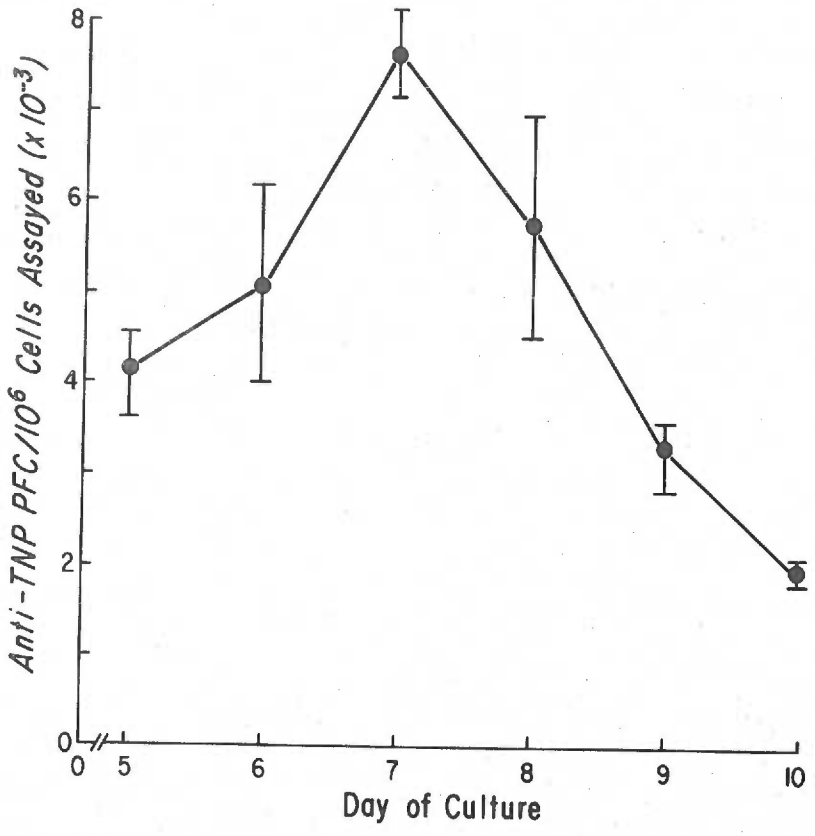

Anti-TNP PFC/10^6 Cells Assayed (x 10^-3)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
5
6
7
8
9
10
Day of Culture

Figure 5. Kinetics of the indirect IgG memory response of primed spleen cells to the optimum immunogenic dose of TNP-T4. Cells were cultured in the presence of 6×10^6 PFU TNP-T4 (PFU/ml) and assayed for indirect PFC on the days indicated. The results are shown as the indirect anti-TNP PFC/10^6 cells assayed ± S.E.M. as in Figure 1. Mice used 24 weeks after priming.


Anti-TNP PFC/10^6 Cells Assayed (x 10^-3)
8
6
4
2
0
0
5
6
7
8
9
10
Day of Culture

Manuscript 2

Independent Precursors for Thymus Dependent and Thymus Independent IgG Memory B Cells

ABSTRACT

Spleen cells from mice primed with the thymus dependent antigen trinitrophenyl keyhole limpet hemocyanin several months earlier can be cultured *in vitro* to give vigorous IgG antihapten PFC responses to thymus dependent and thymus independent forms of the hapten. The IgG memory precursors responding to these two forms of the hapten constitute functionally distinct subpopulations which we have designated as $B_{1\gamma}$ and $B_{2\gamma}$ to represent the precursor cells responding to the thymus independent and thymus dependent antigens respectively. Four types of evidence for these subpopulations are presented 1) the responses to the two types of antigen are additive when both forms are added to the same culture; 2) the precursor frequency for the thymus dependent and thymus independent populations is different although expansion over primary IgM precursor frequencies was not detectable; 3) the avidities of the PFC elicited by each antigen are distinct; the thymus independent antigens elicit lower avidity PFC; 4) selective killing of one population can be accomplished by BUdR and light treatment without affecting the other population.

INTRODUCTION

Several laboratories including our own have reported that IgM precursor B lymphocytes responding to thymus dependent (TD) and thymus independent (TI) forms of the same hapten may represent functionally

distinct subpopulations of B lymphocytes (15,28,32-34). The relationship between these subpopulations is still the subject of speculation, but it has been argued that TI and TD responding B lymphocytes represent immature and mature forms respectively of the same cell lineage (34); however, the evidence for this is circumstantial, and it is also possible that they represent distinct subpopulations with origins at the stem cell stage. Delineation of the ontogenetic relationship between TD and TI responding B lymphocytes is clearly of importance to understanding the developmental patterns of the humoral immune network.

Recently we described *in vitro* IgG secondary responses of large magnitude to TD and TI forms of the trinitrophenyl (TNP) or dinitrophenyl (DNP) haptens in spleen cells from mice primed to the TD antigen trinitrophenyl hemocyanin (TNP-KLH) (214). These results provided *in vitro* confirmation of an *in vivo* study by Braley-Mullen using TD and TI forms of pneumococcal polysaccharide-SIII (26) and emphasized the ability of TI antigens to trigger IgG memory B cells. Here we present evidence to indicate that, like IgM precursors, the precursors of IgG memory also appear to be divisible into functionally distinct subpopulations of TI and TD responding cells. Based on avidity differences in the PFC populations elicited, this functional differentiation may be marked by differences in V region gene expression.

MATERIALS AND METHODS

Mice. Adult female Balb/c mice were obtained from Charles River

Breeding Labs., Willmington, Mass. and were caged in groups of 6 with free access to water and food.

Antigens. Trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) was prepared as described previously (57) and had a mole ratio of TNP_{1067}-KLH. TNP-T4 bacteriophage was prepared as described previously (58). Dinitrophenylated-dextran (DNP-dextran) was a gift from Dr. M. Feldmann.

Immunization. Mice were primed at 2-3 months of age with 3 injections of TNP-KLH-bentonite as previously described (180).

Cell Culture. Spleen cells from at least 3 mice primed 2-4 months previously were pooled and cultured using microtiter plates (186). Culture medium was supplemented with 5×10^{-5}M 2-mercaptoethanol (186) and nucleosides (187). The antigen dose is indicated in the results.

Plaque Assays. Anti-TNP plaque-forming cells (PFC) were detected using TNP-haptenated sheep red blood cells (TNP-SRBC) as prepared previously (185). Cells from 8 replicate microcultures were pooled and plated as one culture for PFC (188). Three such pooled cultures were assayed per experimental point. Cells producing IgM anti-TNP antibody were detected by direct plating. Cells producing IgG anti-TNP antibody were detected by adding goat anti-mouse IgG antiserum (205) and anti-mouse μ chain antiserum to suppress IgM PFC (189).

Avidity Determinations. IgG PFC were assessed as above with TNP-ε-aminocaproic acid incorporated into the plaque assay at various molar concentrations (206). Diluent was added in place of free hapten for controls. Cells at each concentration of inhibitor and controls were plated in quadruplicate with cultured cells diluted so that control

slides had 250-300 PFC per slide.

Selective Suicide. 5 bromouridine deoxyribose (10^{-6} M final concentration, BUdR) was added to cultures 48 hrs after initiation (209). The cultures were illuminated either for 3 hours on day 3 or for 2 hours on days 3 and 4; the latter had a more pronounced effect. After the final illumination the cultures were washed and reincubated with fresh culture medium either with or without additional antigen.

Limiting Dilution Analysis. Precursor frequencies for TD and TI antigens were obtained by culturing cells under conditions where B cells were limiting (33). Sufficient helper function was assured by adding helper primed Mitomycin C treated spleen cells (212). Sixty wells were cultured per experimental point. The number of positive wells was determined by plaque assay of individual wells. The precursor frequency was calculated by the Poisson statistic as in (33).

RESULTS

Previously we have shown that spleen cells from mice primed by three weekly injections of the TD antigen TNP-KLH developed a vigorous anti-TNP IgG response when placed in vitro for 7-9 days with either TD or TI antigens and that the responses to these two forms of the hapten were equal in magnitude (214). At the same time we noted that there was a difference in the relative requirement for 2-mercaptoethanol between the TD and TI initiated responses and that the kinetics of these responses differed. These distinctions led us to ask whether the IgG memory cells responding to TD and TI antigens were different B

lymphocytes as has been shown for the primary IgM response (15,28,32-34).

A simple first experiment was the addition of TD and TI antigens either alone or simultaneously to the same culture. If these antigens were stimulating different B cell subpopulations, the response of the culture challenged simultaneously with both antigens would be expected to equal the sum of the individual responses. Table I shows the results of one of 4 such addition experiments. Spleen cells from TNP-primed mice were challenged with optimum doses of either TNP-T4 or TNP-KLH or both antigens simultaneously. The cells were cultured for 5 or 7 days, harvested and assayed for anti-TNP IgG PFC. On day 5 we observed 8000 PFC/10^6 for both TNP-T4 and TNP-KLH stimulated cultures. If the TD and TI responses were additive as has been shown for primary IgM responses, we would have expected 17,000 PFC/10^6, and we observed 20,000 in the simultaneously challenged culture; as predicted the results were additive. Likewise, on day 7 we observed 22,000 and 12,000 PFC/10^6 cells for TNP-T4 and TNP-KLH stimulated cultures respectively; we expected 35,000 PFC/10^6 based on simple addition, and we observed 37,000 PFC/10^6 in cultures challenged simultaneously with both antigens. Thus addition experiments support the notion that separate subpopulations of IgG memory cell precursors are responding to TD and TI antigens.

Having observed addition by simultaneous challenge with TD and TI antigens, we have carried out preliminary analysis of the frequencies of TD and TI IgG memory precursors. To date we have only evaluated frequencies based on day 5 of culture. Typical results are shown in Table II where the TI precursor frequency is nearly 5 times greater than that of the TD precursors. We have encountered technical

difficulties in getting cultures to persist beyond day 5 in the micro system used for precursor analysis. The total number of precursors in each category may ultimately be shown to be greater as later days of culture are evaluated. The important point, however, is that the number of TI IgG precursors has consistently been larger.

As a third approach we have examined the avidity distribution of the PFC stimulated by TD and TI antigens using hapten inhibition as described by Goidl and Siskind (206). Results typical of a large number of such experiments are shown in Fig. 1. They indicate that, whereas the TI antigen elicits only a narrow range of low avidity PFC (10^{-5} M TNP-ε-amino caproic acid (EACA) was the lowest concentration which inhibited the TI response), the TD antigen elicited a more heterogeneous range of PFC of higher avidity. Furthermore, we can exclude the possibility that the TI antigen selectively blocks the expression of high avidity PFC since when both TD and TI antigens were added to the same culture, both high and low avidity PFC were detected.

Finally, we designed suicide experiments using BUdR and light to eliminate specifically one antigen-responding population prior to stimulation by a second antigen. In these experiments TNP primed spleen cells were cultured with an optimum dose of TNP-T4 or without antigen. BUdR was added to antigen stimulated cultures on day 2 followed by either 3 hours of illumination on day 3 or 2 hours of illumination on days 3 and 4. After the final illumination, the cells were washed, and antigen, either TI or TD, was added and the cells recultured. Typical results are shown in Table III. As can be seen the presence of the TI

antigen prior to BUdR and light treatment greatly reduced the subsequent response to rechallenge with the TI antigen but did not affect the response to the TD antigen TNP-KLH. We have done the reciprocal experiment (i.e. TNP-KLH added first), and have obtained similar results. However, in these latter experiments we have not yet ruled out the possible effect of BUdR on activated helper T cells. The experiments using the TI antigen first, however, clearly indicate that it is possible to abolish the response to the TI antigen without affecting the response to the TD antigen. We have observed in all BUdR experiments that elimination of one responding population increases the response to the unaffected population dramatically. It is possible that this reflects removal of a B cell crowding effect (211) or more likely an elimination of suppressor cells or their precursors which may also be cycling (213) in these cultures and, therefore, susceptible to BUdR treatment. However, we have not yet investigated this aspect.

DISCUSSION

The results reported here for IgG memory precursors are in keeping with those reported previously for IgM precursors (15,28,32-34) and thys lend themselves to the interpretation that B cell precursors responding to thymus dependent and thymus independent antigens represent functionally distinct subpopulations.

Simultaneous addition of TD and TI antigens to the same culture resulted in addition with the response equalling the sum of the responses

to each antigen alone. Although not shown the secondary IgM responses were also additive. In some experiments synergistic responses greater than those expected from simple addition were obtained. Such synergy has also been observed in primary addition experiments of this type (32, 33). Quintans and Cosenza (33) observed synergy in the IgM response only if the TD response was initiated 24 hours before adding the TI antigen and suggested that some TD responsive B cells may have been driven to TI along a common differentiation pathway. On the other hand since we found that synergy could result from simultaneous addition of both TD and TI antigens, we, therefore, suggested that nonspecific T cell factors might be able to affect TI B cells after initial triggering (32). This has remained true in those instances where we have observed synergy in IgG responses. However, the explanation for synergy remains unanswered.

We have found consistently that the TI IgG memory precursors were more numberous than TD precursors. We were surprised, however, to find that the number of precursors was not larger since they are within the range reported previously for IgM precursors (33). We cannot, for the present, state whether these numbers reflect the actual numbers of precursors and that memory has developed in these mice through an unequal division mechanism in which the total memory pool has not expanded or whether it reflects a technical problem. We have succeeded in measuring precursor frequencies only on day 5 in the micro cultures used for limiting dilution analysis (33) and these cultures have not generated the PFC expansion we observe on days 7 and 9 in conventional micro

Mishell-Dutton cultures (214). Thus it is possible that IgG precursors which would not be triggered until later stages of in vitro culture escape detection. The important point here, however, is that the number of precursors differs for the two populations.

Two aspects of the secondary IgG memory response to TD and TI antigens shown here differ from those previously reported for IgM responses. The first is that there are apparent differences in the avidity distribution of PFC stimulated by the TD and TI antigens (Figure 1). We have observed this consistently for IgG memory populations; PFC stimulated by either TNP-T4 or DNP-dextran are of low avidity; whereas a broad spectrum of higher avidity PFC are generated by TNP-KLH. On the contrary no differences in avidity were detected in primary IgM PFC stimulated by TD and TI forms of either TNP (32) or phosphoryl choline (33). Whether this reflects the technical difficulty of measuring relative avidity of IgM responses or a fundamental difference in IgM and IgG precursors cannot be determined at present. However, the results among IgG precursors may be taken as evidence that functional differentiation to TD and TI responsiveness reflects differences in V region gene expression.

The narrow spectrum of avidities found among TI IgG PFC suggests tha the TI antigens are not serving here a polyclonal activators since we sould expect all of the available avidity classes to be expressed in the latter case. The results are in keeping with the view that expression of high avidity PFC reflects a thymus dependent element in avidity maturation (208).

The second way in which this study of TD and TI responding IgG

precursors differs from IgM studies is that we were able to achieve substantial elimination of the TI response with BUdR and light treatment without affecting the TD response upon subsequent addition of antigen. Similar types of experiments utilizing the hot thymidine pulse technique were attempted with the phosphoryl choline IgM system but were inconclusive due to technical problems (33). In this regard we have observed in all of our BUdR experiments that secondary IgM precursors are considerably less sensitive to this treatment than IgG precursors although selective killing is still observed (not shown). The results of these suicide experiments constitute a powerful argument for functionally distinct subpopulations but do not allow us to draw a conclusion concerning their developmental relationship.

It appears from several types of evidence then that there are functionally differentiated subpopulations of B lymphocytes which differ in their ability to read the hapten carrier complex and to distinguish those which are thymus independent from those which are thymus dependent. These subpopulations were originally termed B_1 and B_2, respectively, by Playfair and Purves (28). Two hypotheses for their ontogenetic development are shown in Fig. 2 which is based on the model of Cambier *et al.* (34) who suggested a common pathway of development from stem cell → B_1 → B_2 based on properties which B_1 cells in the adult have in common with immature neonatal B cells. The alternative view proposed by Quintans and Cosenza (33) is that B_2 cells may be precursors of B_1. Obviously it is also possible that B_1 and B_2 are "separate categories" of cells as suggested recently by Kincade (161) who found CBA/N mice to

be devoid of colony-forming B cells (immature, B_1?). Since CBA/N mice do have adequate numbers of B_2 cells, they respond to TD antigens but poorly, if at all, to TI antigens (207,210), they do not fit readily into the $B_1 \rightarrow B_2$ model. Our demonstration of $B_{1\gamma}$ and $B_{2\gamma}$ memory populations further complicates this picture; they could arise from $B_{1\mu}$ and $B_{2\mu}$, respectively, if the separate lineage hypothesis is correct or from a common precursor. If the latter were true, we would favor $B_{2\mu}$ as the immediate precursor since it was the TD antigen which primed for the subsequent TI IgG response. Clearly it it too early to predict which of these ontogenetic schemes is correct. Furthermore, Kimoto et al. (204) recently reported obtaining secondary IgE responses to both DNP-ovalbumin and DNP-ficoll. Although they did no analysis for independent TD and TI subpopulations, we can expect that similar subpopulations probably exist for all Ig classes.

Presumably the development of these functionally distinct subpopulations during the course of evolution was subjected to a selective pressure which ensured their survival. Perhaps the selective rationale will become apparent once the develpmental relationships between TD and TI responding populations of B cells from virgin precursor to memory population is delineated. Thus delineation of these relationships should remain an important topic for the foreseeable future.

TABLE I

Addition of IgG Responses on Double Challenge with TD and TI Antigens

	Anti-TNP PFC/10^6	
	Days in Culture	
Antigen	5	7
TNP-T4[a] (TI)	8519 ± 606[b]	22771 ± 2097
TNP-KLH (TD)	8504 ± 1407	12298 ± 291
TNP-T4 + TNP-KLH	20691 ± 96	37264 ± 6537
Expected if Additive	17023	35069

[a] Antigen doses were optimal: TNP-T4 (6 x 10^6 PFU/ml), TNP-KLH (0.02 µg/ml).

[b] IgG PFC enumerated in the presence of a suppressive amount of anti-IgM antiserum. Mean ± standard error.

TABLE II

Frequency of TD and TI IgG Precursors

Antigen	IgG Precursors/10^5 Spleen Cells[a]
TNP-T4[b] (TI)	1.2[c]
TNP-KLH (TD)	0.26

[a] Assayed on day 5 of culture.

[b] Optimum doses: TNP-T4 (1.5×10^6 PFU/ml), TNP-KLH (0.002 µg/ml).

[c] Calculated from Poisson statistic as in (187) using 60 cultures per dilution point in limiting dilution analysis.

TABLE III

Selective Killing of TI IgG Precursors by BUdR + Light[a]

Challenged with TNP-T4	% of Control Response			
Rechallenged with[b]	Exp 1[c]	Exp 2	Exp 3	Exp 4
TNP-T4 (TI)	37	21	8	4
DNP-Dextran (TI)	nd	nd	13	1
TNP-KLH (TD)	249	186	192	380

[a] Cells were challenged with TNP-T4 on day 0 of culture. BUdR (1×10^{-6} M final concentration) was added on day 2. In Exp 1 and 2 the cells were illuminated 24 hrs later for 3 hrs. In Exp 3 and 4 the cells were illuminated 24 and 48 hrs later for 2 hrs on each day. Immediately after illumination the cells were centrifuged, washed and rechallenged with the TD or TI antigens as indicated.

[b] Antigen doses were optimal: TNP-T4 (1.5×10^6 PFU/ml), DNP-dextran (0.01 µg/ml), TNP-KLH (0.002 µg/ml).

[c] Assayed 5 days (Exp 1,2,3) or 7 days (Exp 4) after rechallenge. The control cultures were treated with diluent in place of BUdR and illuminated, washed and challenged with antigen as the experimental groups.

Figure 1. Avidity of IgG PFC generated by TD or TI antigens in vitro. The inhibitor TNP-EACA in varying molar concentrations was added to the plaque assay and compared to controls in which diluent was added in place of inhibitor. The assay contained anti-IgM antiserum to suppress IgM PFC as well as anti-IgG antiserum and complement. Cells were harvested on day 5 of culture.

The abscissa represents $\log_{10}$ of the inverse of the free hapten concentration used as inhibitor. The ordinate represents the percentage of the total population of PFC which were inhibited. Avidity increases to the right. The antigen used to stimulate the in vitro response is shown at the top. Optimal concentrations of antigen were used: TNP-T4 (6×10^6 PFU/ml), TNP-KLH (0.002 µg/ml).

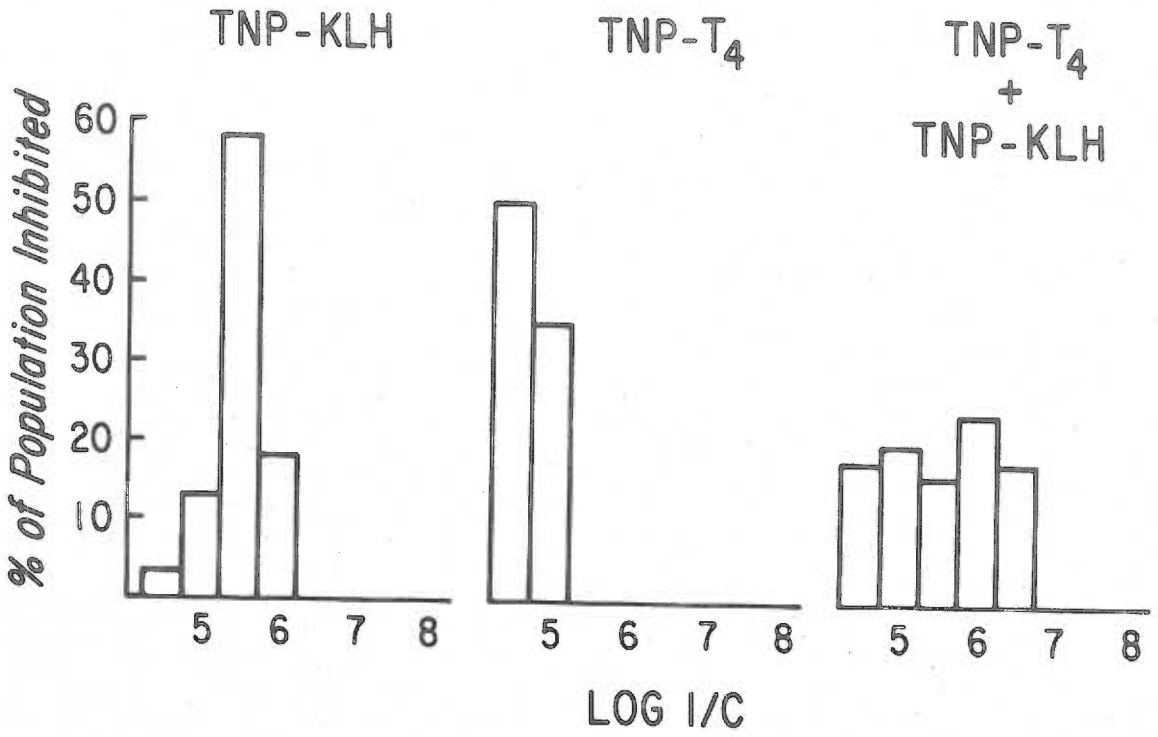
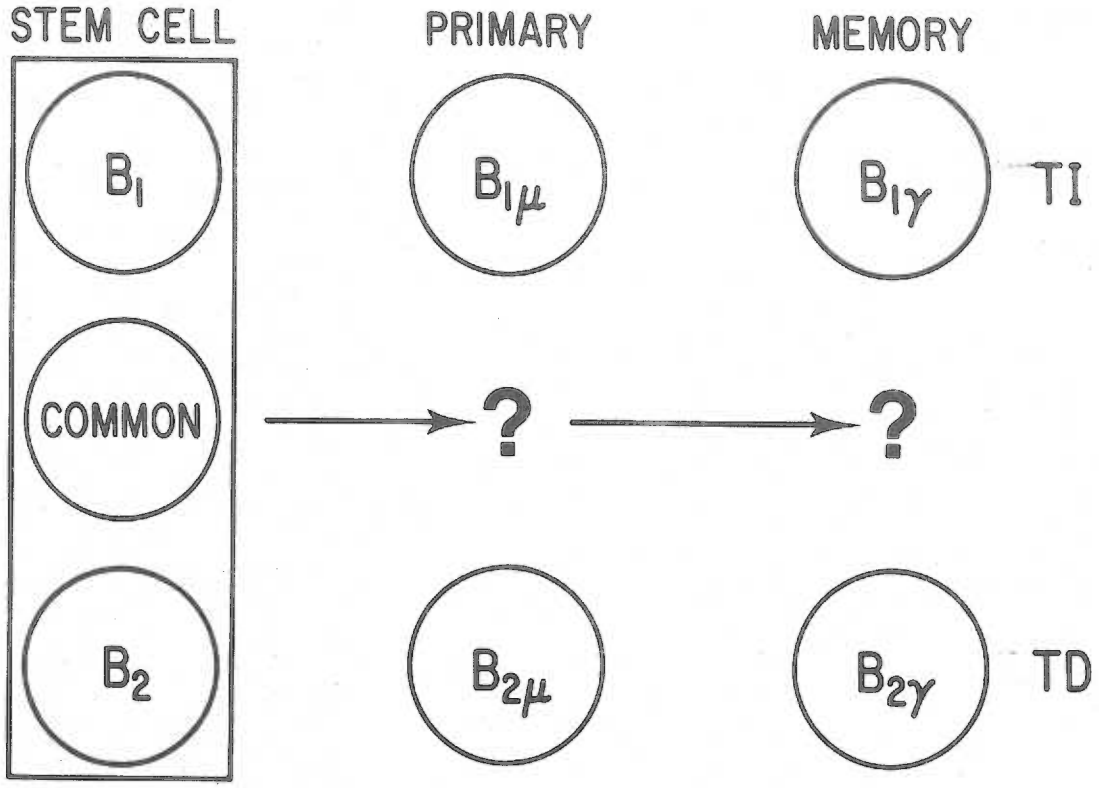

TNP-KLH
TNP-T4
TNP-T4
+
TNP-KLH
% of Population Inhibited
60
50
40
30
20
10
5
6
7
8
LOG I/C

Figure 2. Functional differentiation among primary and memory B lymphocyte precursors.


STEM CELL
PRIMARY
MEMORY
B_1
$B_{1\mu}$
$B_{1\gamma}$
TI
COMMON
?
?
B_2
$B_{2\mu}$
$B_{2\gamma}$
TD

Manuscript 3

Distinct Subpopulations of IgG Memory B Cells Respond to Different Molecular Forms of the Same Hapten

ABSTRACT

Spleen cells from mice primed with the thymus dependent antigen trinitrophenyl keyhole limpet hemocyanin several months earlier can be cultured in vitro to give vigorous IgG antihapten PFC responses to thymus dependent (TD) and thymus independent (TI) forms of the same hapten. Here we show that the IgG memory precursors which respond to these 2 forms of the hapten constitute functionally distinct subpopulations. We have designated these subpopulations as $B_{1\gamma}$ and $B_{2\gamma}$ to represent secondary precursor cells responding to TI and TD antigens, respectively. Three types of evidence for these subpopulations are presented: 1) In vitro secondary IgG responses to TD and TI forms of the TNP hapten are additive when both forms are added to the same culture. 2) The precursor frequencies for the TD and TI antigens are additive, but addition is not observed between two TD or two TI antigens. 3) Each population can be selectively eliminated by BUdR and light treatment without affecting the other population. The ontogenetic relationships between these subpopulations are discussed in relation to all presently proposed subpopulations $B_{1\mu}$, $B_{2\mu}$, $B_{1\gamma}$ and $B_{2\gamma}$.

INTRODUCTION

Recent interest has focused on the question of specific subsets of B lymphocytes responding to different macromolecular forms of the same haptenic determinant. We and others have suggested that IgM precursor

B cells which respond to thymus independent (TI) and thymus dependent (TD) antigens represent functionally distinct subpopulations termed B_1 and B_2 respectively (15,28-35). This conclusion is based on additivity in the immune response to simultaneous challenge with TD and TI forms of hapten (28,32), similar additivity in the precursor frequencies of TD and TI responding B cells (33-35), differences among B cells in susceptibility to tolerance induction in vivo and in vitro (29,32,34) and partial physical separation or enrichment of TD and TI responding lymphocytes (15,31).

The comparable sensitivity to tolerance induction between B_1 cells and immature B cells in neonatal animals has led to the suggestion that B_1 cells are the early stage in a developmental sequence, $B_1 \rightarrow B_2$ (30, 34). However, not all experimental facts fit this scheme, and both the sequence $B_2 \rightarrow B_1$ (33) and separate lineages for B_1 and B_2 (161) have been proposed.

We recently described in vitro IgG secondary responses to TD and TI forms of the trinitrophenyl (TNP) or dinitrophenyl (DNP) haptens in spleen cells from mice primed with the TD antigen TNP-keyhole limpet hemocyanin (TNP-KLH) (214). Differences observed in the kinetics and 2 mercaptoethanol dependence of these responses suggested that IgG memory B cells might also be divisible into TI and TD subpopulations as is the case for IgM B cells. Here we show that like IgM responses the IgD TD and TI plaque-forming cell (PFC) responses are additive as are their precursor frequencies. In addition, we show that unlike the IgM responses which have been reported (33) the IgG subpopulations

can be discriminated between on the basis of selective suicide with bromouridine deoxyribose (BUdR) and light treatment following antigenic stimulation. Consequently, we have proposed the designations $B_{1\mu}$ and $B_{1\gamma}$ and $B_{2\mu}$ and $B_{2\gamma}$ to distinguish the primary and secondary forms of these TI and TD populations respectively, and we argue that B_1 and $B_{2\gamma}$ need to be accounted for in any ontogenetic scheme of B cell maturation.

MATERIALS AND METHODS

Animals. Adult female Balb/C mice were obtained from Charles River Breeding Labs., Willmington, Mass. and were caged in groups of 6 with free access to food and water. They were used when 2-10 months of age. Juvenile female Balb/C mice were obtained from our breeding colony and were used when 1 month old.

Antigens. Trinitrophenylated-T_4 bacteriophage (TNP-T_4) was prepared as previously described (58). Keyhole limpet (KLH) and horseshoe crab (HCH) hemocyanins were trinitrophenylated according to the method of Rittenberg and Amkraut (57) and had mole ratios of TNP_{1057}-KLH and TNP_{788}-HCH assuming the mol wt of each protein = 8×10^6. Dinitrophenylated dextran ($DNP_{5.7}$-dextran) and $DNP_{3.9}$-ficoll were gifts from Dr. M. Feldman. HCH was a gift from Dr. A. Malley.

Immunizations. TNP-KLH and HCH were made particulate by coating on to bentonite (Fisher Scientific) according to the method of Gallily and Garvey (216) as modified previously (185). Mice were given intraperitoneal injections of protein-bentonite (T-K-B or HCH-B) every week

for a total of 3 weeks. Each injection contained 100 µg protein in 0.5 ml saline. Double carrier primed mice were obtained by injecting T-K-B primed mice with HCH-B.

Cell Cultures. Spleen cells were cultured in micro Mishell-Dutton cultures (186) as described previously (214). Each microculture contained 10^6 cells/well and the contents of 8 such wells were pooled for PFC assay as one culture. Three such cultures were assayed per experimental point to obtain data for statistical analysis.

Limiting dilution experiments were performed according to Quintans and Cosenza (33) using Falcon No. 3034 tissue culture trays. In these experiments normal syngeneic thymocytes (3×10^4/well, optimum) were added as filler cells (211,217). In addition mitomycin-C treated (Sigma Chemical Co., St. Louis, Mo. 25 µg/ml for 30 min at 37° C) carrier-primed spleen cells (3×10^4/well, optimum) were added as a source of helper T cells (212). The number of responding cultures was assayed using two methods: 1) At the end of each culture period the trays were centrifuged for 15 min at 500-1000 g, and the tray was flooded with cold Hank's balanced salt solution to wash out the culture fluid. The cells in each well were resuspended in 0.1 ml of plaquing mixture (see below) and used in a plaque assay against TNP-haptenated sheep red blood cells (TNP-SRBC). 2) The culture supernatant from each well was tested for anti-TNP IgG antibody by a modification of the anti-TNP hemolytic spot test used previously (180, see below).

Detection of anti-TNP response. Cells from regular microcultures secreting anti-TNP antibody were detected by plaque assay using TNP-SRBC (185) and the slide technique of Cunningham and Szenberg (188).

Cells producing IgM anti-TNP antibody were detected by direct plaquing with guinea pig serum (1:3) as a source of complement. Cells producing IgG anti-TNP antibody were detected by adding goat anti-mouse IgG antiserum and complement. The latter assay was conducted in the presence of sheep anti-MOPC 104E (IgM, λ) antiserum which suppressed IgM plaques by ≥ 95% (189). The anti-MOPC 104E was kindly provided by Dr. A. Malley.

IgG PFC from limiting dilution cultures were detected by adding the contents of each well to 0.1 ml of plaquing medium consisting of Eagle's minimum essential medium (MEM) containing guinea pig complement (1:10), goat anti-mouse IgG, sheep anti-MOPC 104E and TNP-SRBC. These mixtures representing individual wells were then plated in Cunningham-Szenberg chambers. For the hemolytic spot test of supernatants from limiting dilution cultures, 5 µl of a 1:20 dilution of anti-MOPC 104E was added to each well and allowed to react for 5-10 min at room temperature. This prevented any IgM hemolytic spots from appearing. Next 5 µl from individual wells was spotted on an agarose surface (0.7%) containing 0.5% TNP-SRBC. The liquid was allowed to dry for 1 hr at 37°C, the plates were flooded with 2 ml of a 1:20 dilution of goat anti-mouse IgG antiserum and reincubated for 30 min at 37°C. Unabsorbed anti-IgG antiserum was removed and 2 ml of a 1:6 dilution of guinea pig serum was added and the plates incubated for 1 hour at 37°C.

Selective Suicide. IgG memory cells responding to either TD or TI antigens were selectively killed by using a UV modification (175) of the 5-bromouridine deoxyribose (BUdR) method of Zoschke and Bach (209).

Briefly, 10^{-6} M BUdR (Sigma Chemical Co., St. Louis, Mo.) was added to regular microcultures challenged two days previously with a TD or TI antigen. The cultures were illuminated for 2 hours each on days three and four by placing them on ice 3-6 cm from a germicidal UV lamp (Sylvania) at 6000 ergs/cm^2. After the final illuminations the plates were centrifuged at 1000 g for 15 min, the supernatant was removed and the cultures washed and reincubated with fresh medium either with or without additional antigen.

T Cell Depletion. Washed spleen cells from TNP primed mice were suspended at 2 X 10^7 cells/ml in MEM containing 0.3% bovine serum albumin (MEM-BSA). Rabbit anti-mouse brain antiserum (Anti-θ, Cedarlane Laboratories, London, Ontario, Canada, Lot 220) was added at a final dilution of 1:12 and the cells were held at 4°C for one hour. The cells were then pelleted by centrifugation, brought up to the original volume with MEM-BSA containing 7.5% rabbit complement (Low Tox Lot 4012, Cedarlane Laboratories) and incubated for an additional 45 min at 37°. The cells were then washed and brought up to the number of cells indicated (See Results).

Poisson Analysis of Limiting Dilution Data. When limiting dilution experiments were analyzed by plaquing individual wells and scoring for anti-TNP PFC, a well was arbitrarily defined as positive if it contained at least two PFC and a central lymphocyte could be confirmed under higher magnification. For hemolytic spot test analysis a well was defined as positive if the supernatant produced a zone of hemolysis in the red cell layer. The precursor frequency was calculated by Poisson statistic from

the fraction of non-responding cultures using the relationship; $P(0) = e^{-n\lambda}$ - where P(0) is the fraction of negative cultures, n is the number of T-K-B primed spleen cells per well, and λ the precursor frequency (35).

The precursor frequencies shown in Table I were derived from analysis of triplicate 60-well culture plates per point. The resultant data were analyzed for significance using two-way analysis of variance and a standard F test to determine probability (218).

RESULTS

Effect of In Vitro Challenge with Both TD and TI Antigens.

a) Plaque forming cell responses. We reported previously that spleen cells from mice primed by three weekly injections of the TD antigen TNP-KLH were able to develop an anti-TNP IgG response when challenged in vitro with either TD or TI antigen (214). Differences in the relative requirement for 2-mercaptoethanol between TD and TI initiated responses as well as in the kinetics of these responses suggested that they could involve different subpopulations of IgG precursors in accord with recent reports of such subpopulations among IgM precursors (15,28-35).

As was done previously with IgM responses (32), we added TD and TI antigens either alone or simultaneously to the same culture. If these antigens were stimulating different B cell subpopulations, the response to simultaneous challenge with both antigens would be expected to be

additive. Figures 1 and 2 show the results of 1 of 5 such addition experiments. Spleen cells from TNP rpimed mice were challenged with optimum doses of either TNP-T_4 or TNP-KLH or both antigens simultaneously. The cells were cultured for 5 or 7 days and then harvested and assayed for IgM (Fig. 1) and IgG (Fig. 2) anti-TNP PFC. It can be seen that either antigen alone stimulated significant IgM and IgG responses. When a mixture of the TD and TI antigens was added to the same culture, the response was approximately equal to the sum of the responses to each antigen alone in the case of IgG and was synergistic on day 5 of the IgM response. Synergy rather than addition has also been observed previously in the primary IgM response to TNP (32) and to phosphoryl-choline (33). Synergy is occasional rather than the rule, and we have also observed it in IgG responses (not shown). We attribute it to accessory cell help suggesting that B cells once triggered by a TI antigen may benefit from contact with factors derived from a concurrent TD response (32). However, this aspect has not been investigated. As reported previously for primary IgM responses (32), additive IgG responses could not be obtained by using higher doses of one antigen alone; in fact, higher than optimum doses of TNP-KLH are suppressive (180,214). We have not detected addition on day 9 of culture consistently probably because the TI response usually declines rapidly after day 7 (214).

b) Precursor Frequency Analysis. In view of the additive nature of the IgG responses to simultaneous challenge with TD and TI antigens, we carried out limiting dilution experiments to determine if the TD and TI IgG precursor frequencies were additive as has been shown for IgM precursors (33,35).

Results of four typical experiments are shown in Table I. The number of positive wells was determined by hemolytic spot test for IgG anti-TNP antibody after 5 days of culture. Addition of precursor frequencies was only observed when TD and TI antigens were added simultaneously ($p<0.01$ Exp. 1 and 4) but not when cultures were simultaneously challenged with two TD ($p>0.05$ Exp. 2) or two TI ($p>0.05$ Exp. 3) antigens. Eight other experiments were conducted (not shown) in which TNP-T_4, DNP-ficoll, TNP-KLH and TNP-HCH were used in all possible combinations in the same experiment. The size of the experiments precluded utilization of triplicate cultures. Nevertheless, the results were similar to those shown here with only combinations of TD and TI antigens showing additive IgG precursor frequencies. These results are consistent with the hypothesis that TD and TI antigens stimulate different IgG memory precursors. The absolute frequencies of anti-TNP IgG precursors do not differ markedly from those obtained previously for IgM precursors by similar *in vitro* methods (35). The highest frequency we have observed in limiting dilution analysis for IgG precursors was $2.1/10^5$ in response to TNP-KLH + TNP-T_4. These values for IgG precursors are slightly higher than those reported for secondary anti-DNP IgG obtained from *in vivo* limiting dilution analysis (164).

Clonal Expansion of IgG Precursor Cells. Recently it was suggested that IgM-producing B cells responding to TD antigens tend to proliferate to a greater extent than those responding to TI antigens (33,35). We have begun to analyze the burst characteristics of individual IgG precursors in the same way. The cells were plated as in limiting dilution

analysis at low B cell frequency so that at least 37% of all wells were negative indicating, according to the Poisson distribution, that each well contained no more than one precursor. The results of one of two such experiments are shown in Fig. 3 in which the variation in burst size per individual precursor is plotted as a distribution of the population on days 5, 7 and 9 of culture. It can be seen that burst sizes of 64 or greater were not detected until day 7 and that these tended to be in response to the TD antigen; thus on days 7 and 9, respectively, 43 and 50% of the TD response consisted of large bursts (≥64); whereas, on the same days less than 20% of the response to either TI antigen was large. As with conventional microcultures, the TI response diminishes rapdily after day 7 (214) so that by day 9 a large percentage of the TI response shows small burst characteristics. This type of analysis on relatively small numbers of individual cells should be viewed as preliminary until a much larger number has been examined, but at present these results appear similar to those reported for IgM precursors.

Selective Killing of TI or TD IgG Precursors of BUdR and Light. If TD and TI IgG memory B cells do constitute functionally distinct subpopulations, then it should be possible to kill one population selectively with BUdR and light prior to stimulation with the second antigen. If selective killing were achieved, the cultures should respond to secondary challenge with the heterologous form of antigen but not to the homologous form. In these experiments TNP primed spleen cells were first cultured with an optimum dose of TNP-T_4 (Table II) or TNP-KLH (Table III) or without antigen. BUdR was then added on day 2 followed by 2 hours of illumination on days 3 and 4. After the final

illumination, the cells were washed in situ, and antigen, either TD or TI in fresh medium was added and the cells recultured. Results showing selective killing of TI IgG precursors are shown in Table II and represent one of 5 experiments in which similar results were obtained. Cells given TNP-T_4 followed by BUdR and light could not respond to the TI antigens TNP-T_4 or DNP-dextran (99% killing); whereas their response to TNP-KLH was only slightly reduced (6200 PFC/10^6 in controls and 4500 PFC/10^6 in treated cultures).

Selective killing to TD IgG precursors was likewise achieved in reciprocal experiments (TD antigen before BUdR). Although it has been shown that helper T cells are unaffected by 700-1600 rads of x irradiation (219,220) or by mitomycin C treatment (212), it was, nevertheless, possible to explain the results of the latter experiments by loss of activated helper T cells rather than B cells. Consequently, the basic experiment was modified so that fresh B cells were added back to a portion of the cells after BUdR and light treatment to show that helper cells were still available in the treated population. Two such experiments are shown in Table III (2 of 6 experiments). In both experiments killing of the TD population had no effect on the response to the TI forms of the hapten TNP-T_4 or DNP-dextran; while the cultures lost the ability to respond to the homologous TD form of the hapten. Furthermore, addition of 10^5 anti-θ and complement treated spleen cells to the TD B-cell-depleted cultures restored their ability to respond to a TD antigen. This indicates that there was a sufficient number of helper T cells in the BUdR treated cultures to generate a response and that the inability to respond was most likely due to selective loss of TD-responding

B cells. We conclude from these experiments that the TD and TI forms of the hapten stimulate different IgG B cell subpopulations each of which can be eliminated by BUdR and light treatment after exposure to their respective antigen forms without affecting the other population.

DISCUSSION

We and others have suggested that TI and TD antigens stimulate different IgM B cell subpopulations called B_1 and B_2, respectively (15, 28-35). The evidence presented here strongly supports the notion that this is also the case for IgG memory B cells. To distinguish between these subpopulations, we shall use $B_{1\mu}$ and $B_{2\mu}$ when referring to IgM-secreting B_1 and B_2 cells and $B_{1\gamma}$ and $B_{2\gamma}$ when referring to IgG-secreting B_1 and B_2 subpopulations.

Simultaneous addition of TD and TI antigens to the same culture resulted in addition in both secondary IgM and IgG responses. By using limiting dilution analysis in which TNP primed spleen cells were limiting, we were able to show that addition of IgG precursor frequencies, like IgM, occurred only when TD and TI antigens were used together and not when two TD or two TI antigens were used (Table I). This has been true also in IgM responses (35). There is one important exception to this rule in that Lewis and Goodman (35) recently reported for IgM precursors that TNP-LPS (TI) was not additive with TNP-horse RBC (TD) but was additive with TNP-ficoll (TI). Preliminary evidence suggests that TNP-LPS is additive with TNP-ficoll but not TNP-KLH in its stimulation of

IgG precursors also (Tittle and Rittenberg, unpublished observations). These results are in agreement with the view that there are different categories of TI antigens (54). In addition, they suggest that TNP-LPS stimulates a B_2 population in a T independent fashion. Thus, perhaps it should be emphasized that thymus dependency or independency of antigens refers to heterogeneous molecular conformations which for unknown reasons require more or less thymic help in eliciting B cell responses. It may only be fortuitous that these heterogeneous conformers allow us to recognize functionally distinct subpopulations and that the significance of these subpopulations lies less with their degree of thymus dependency than with their recognition of various molecular conformations. Nonetheless for the present it appears convenient to retain this nomenclature because of the functional nature of the antigens.

Thymocyte filler cells were absolutely required for extension of the culture period from day 5 to 9 for limiting dilution analysis. During this time the number of progeny cells, or burst size, increased. This corresponds to the delay in the peak *in vitro* IgG responses which we have previously reported (214) and further emphasizes the requirement for a multiplicity of proliferative cycles which has been suggested from mitogen studies to be a characteristic of IgG memory cells (175). Despite this requirement we have observed consistently that precursor frequencies for $B_{1\gamma}$ and $B_{2\gamma}$ are approximately equal; although one may exceed the other in a particular experiment, it seems clear that $B_{1\gamma}$ can constitute a major fraction of the total IgG memory population.

An important difference in results between this study of TD and TI precursors and previous studies of IgM precursors is that we were able

to achieve selective elimination of either the TD or TI response with BUdR and light treatment. Quintans and Cosenza attempted similar experiments using the hot thymidine pulse technique on TD and TI IgM responses to phosphorylcholine (33). Their results were inconclusive due to technical failure. In this regard we have observed in all of our BUdR experiments that secondary IgM precursors are considerably less sensitive to this treatment than IgG precursors although selective killing was still observed (not shown). The results of these suicide experiments constitute a powerful argument for functionally distinct subpopulations.

An interesting observation in these experiments was that cells maintained in culture for four days without antigen but given BUdR and light treatment responded 5-10 times higher when subsequently given antigen than corresponding non-BUdR and light treated cells cultured for the same length of time prior to antigen challenge (not shown). This was similar for both TD and TI responses and did not influence interpretation of these experiments since the comparisons always involved BUdR and light treated cultures. A similar phenomenon has been reported by Zauderer and Askonas (175) who observed an increase in ^{3}H-thymidine incorporation in response to LPS after BUdR and UV light treatment. They ascribed this to the killing of a suppressor population. Indeed, the work of Sy et al. (213) suggests that suppressor cells normally cycle and would thus be susceptible to elimination by BUdR and light by other Class II cytotoxic reagents which kill dividing cells (221). While there was a difference in cell recovery between BUdR and non-BUdR treated cultures, this appeared to have no influence on the interpretation either since although 34-50 percent fewer cells were recovered

following BUdR treatment, the number of PFC/culture was 3-5 fold higher.

We have also found that the TI antigens tend to trigger lower avidity IgG PFC than do TD antigens in these cultures (215). This is in keeping with the view that T cells are required for the development of high avidity memory (208) but also suggests that B_1 and B_2 may not contain identical populations with respect to expression of V region combinations specifying reactivity for a particular antigenic determinant.

Three hypotheses for the ontogenic development of B_1 and B_2 subpopulations have been proposed. Cambier et al. (34) suggested a common pathway of development from stem cell $\rightarrow\rightarrow B_{1\mu} \rightarrow B_{2\mu}$ based on properties which B_1 cells in the adult have in common with immature neonatal B cells. The alternative view proposed by Quintans and Cosenza (33) is that $B_{2\mu}$ cells may be precursors of $B_{1\mu}$ cells. The third possibility is that B_1 and B_2 are separate lineages of cells as suggested recently by Kincade (161) who found CBA/N mice to be devoid of colony-forming B cells (immature, B_1?). Since CBA/N mice do have adequate numbers of B_2 cells, they can respond to TD antigens but poorly to TI antigens (207,210). Consequently, CBA/N mice do not fit readily into the $B_1 \rightarrow B_2$ model nor do wild-type CBA mice; Howard and Hale (157) found that while 14 day old CBA mice respond at 91% of the adult level to DNP-KLH, their response to DNP-ficoll is only 7% of the adults and they only develop the adult's capacity to respond to the TI antigen sometime later.

Recently it was reported that CBA/N mice can respond to TNP-LPS and TNP-Brucella abortus (222). These workers suggested elsewhere (54) that such antigens stimulate a less mature B cell population than other TI antigens such as ficoll, dextran and pneumococcal polysaccharide.

Since, as indicated above, it is possible that TNP-LPS stimulates B_2 cells (35), it is also possible that TNP-Brucella abortus stimulates B_2 cells. Taken together these data could suggest that $B_2 \rightarrow B_1$. However, the demonstration of $B_{1\gamma}$ and $B_{2\gamma}$ memory populations further complicates this picture; they would arise from $B_{1\mu}$ and $B_{2\mu}$, respectively, if the separate lineage hypothesis is correct or from a common precursor. If the latter were true, we would favor $B_{2\mu}$ as the immediate precursor since it was the TD antigen which primed for the subsequent TI IgG response although priming could occur prior to differentiation into B_1 and B_2. Clearly it is too early to predict which ontogenetic scheme is correct.

Finally, Kimoto, et al. (204) recently reported obtaining secondary IgE responses to both DNP-ovalbumin and DNP-ficoll. Although they did no analysis for independent TD and TI subpopulations, their results lead us to predict the existence of $B_{1\varepsilon}$ and $B_{2\varepsilon}$ and that similar subpopulations probably exist for all Ig-secreting cells.

TABLE I

Addition of TD and TI IgG Precursor Frequencies[a]

Experiment	Antigen[b]	IgG precursor frequency[c]/10^5 spleen cells ± SD	
1	TNP-T_4 (TI)	0.48 ± 0.03	
	TNP-KLH (TD)	0.73 ± 0.06	
	Both	1.59 ± 0.28	$p<0.01$[e]
2[d]	TNP-KLH (TD)	1.67 ± 0.08	
	TNP-HCH (TD)	1.37 ± 0.27	
	Both	1.55 ± 0.33	NS
3	TNP-T_4 (TI)	0.88 ± 0.12	
	DNP-ficoll (TI)	0.88 ± 0.09	
	Both	0.81 ± 0.21	NS
4	DNP-ficoll (TI)	0.78 ± 0.10	
	TNP-KLH (TD)	1.18 ± 0.10	
	Both	1.59 ± 0.08	$p<0.01$

a Assayed on day 5 of culture

b Optimum doses of TNP-T_4 (1.5×10^6 PFU/ml), DNP-ficoll (0.001 µg/ml), TNP-KLH (0.002 µg/ml), TNP-HCH (0.002 µg/ml) were used. Cultures contained 3×10^4 normal thymocytes per well in addition to 3×10^4 mitomycin treated KLH primed spleen cells as a source of helper T cells.

c Calculated from Poisson statistic. Each value represents mean ± SD of three cultures with each culture being a plate of 60 culture wells assayed individually per dilution point.

d Used mitomycin treated TNP-KLH-HCH (double carrier) primed spleen cells as helper cells. These cells did not respond when cultured with antigen alone.

e Differs significantly ($p<0.01$) from either antigen used independently when compared either individually or as a group by two-way analysis of variance. NS, not significant ($p>0.05$).

TABLE II

Selective Killing to TI IgG Precursors by BUdR and Light

Antigen Before BUdR and Light	Antigen After BUdR and Light	Anti-TNP IgG PFC/10^6 Cells[b]	% of Control Response
None[a]	TNP-T_4	842 ± 118	100
TNP-T_4	TNP-T_4	2 ± 2	<1
None	DNP-Dex	317 ± 137	100
TNP-T_4	DNP-Dex	0	0
None	TNP-KLH	6200 ± 890	100
TNP-T_4	TNP-KLH	4525 ± 1520	73

a Cells were given no antigen or TNP-T_4 on day 0 of culture. BUdR (1×10^{-6} M final concentration) was added on day 2 to all cultures and the cells were illuminated 24 and 48 hours later for 2 hours on each day. Immediately after illumination the cells were centrifuged, washed and rechallenged with TD or TI antigens as indicated. Antigen doses were optimal: TNP-T_4 (1.5×10^6 PFU/ml), DNP-dextran (0.01 µg/ml), TNP-KLH (0.002 µg/ml).

b Assayed 7 days after rechallenge. Mean of triplicate cultures ± standard error. Each culture represents pooled cells from 8 microculture wells. IgG PFC were obtained in the presence of a suppressive amount of anti-IgM antiserum.

TABLE III

Selective Killing of TD IgG Precursors by BUdR and Light

Antigen Before BUdR and Light	Antigen After BUdR and Light	Anti-TNP-PFC/10^6 Cells Exp. 1	Exp. 2
None[a]	TNP-T_4	848 ± 26	686 ± 36
TNP-KLH	TNP-T_4	1043 ± 241 (123)[b]	744 ± 44 (108)
None	DNP-Dex	nd	454 ± 44
TNP-KLH	DNP-Dex	nd	857 ± 136 (189)
None	TNP-KLH	1796 ± 113	188 ± 6
TNP-KLH	TNP-KLH	0 (0)	4 ± 4 (2)
TNP-KLH	TNP-KLH + 10^5[c] anti-θ treated spleen cells	1233 ± 132	390 ± 22

a Cells were given no antigen or TNP-KLH on day 0 of culture. Cells were then treated as described in Table II. Antigen doses were optimal: TNP-T_4 (1.5 X 10^6 PFU/ml), DNP-dextran (0.01 µg/ml), TNP-KLH (0.002 µg/ml).

b Mean standard error as in Table II. The percent of control response is indicated in parenthesis. nd = not done.

c 10^5 anti-θ treated spleen cells cultured with TNP-KLH but not added to BUdR treated cells gave 0 and 8 ± 8 PFC/10^6 for Exp. 1 and 2 respectively 10^6 anti-θ treated spleen cells cultured with TNP-KLH gave 0 PFC/10^6 for both Exp. 1 and 2.

Figure 1. Addition of secondary IgM anti-TNP-PFC response. TNP-primed spleen cells were cultured for 5 or 7 days with optimum doses to TNP-T_4 (6 X 10^6 PFU/ml), TNP-KLH (0.002 µg/ml) or both antigens simultaneously, then harvested and assayed as described in the text. Each histogram represents the mean ± S.E.M. of triplicate cultures assayed for IgM anti-TNP PFC. The response of cells cultured without antigen has been subtracted.

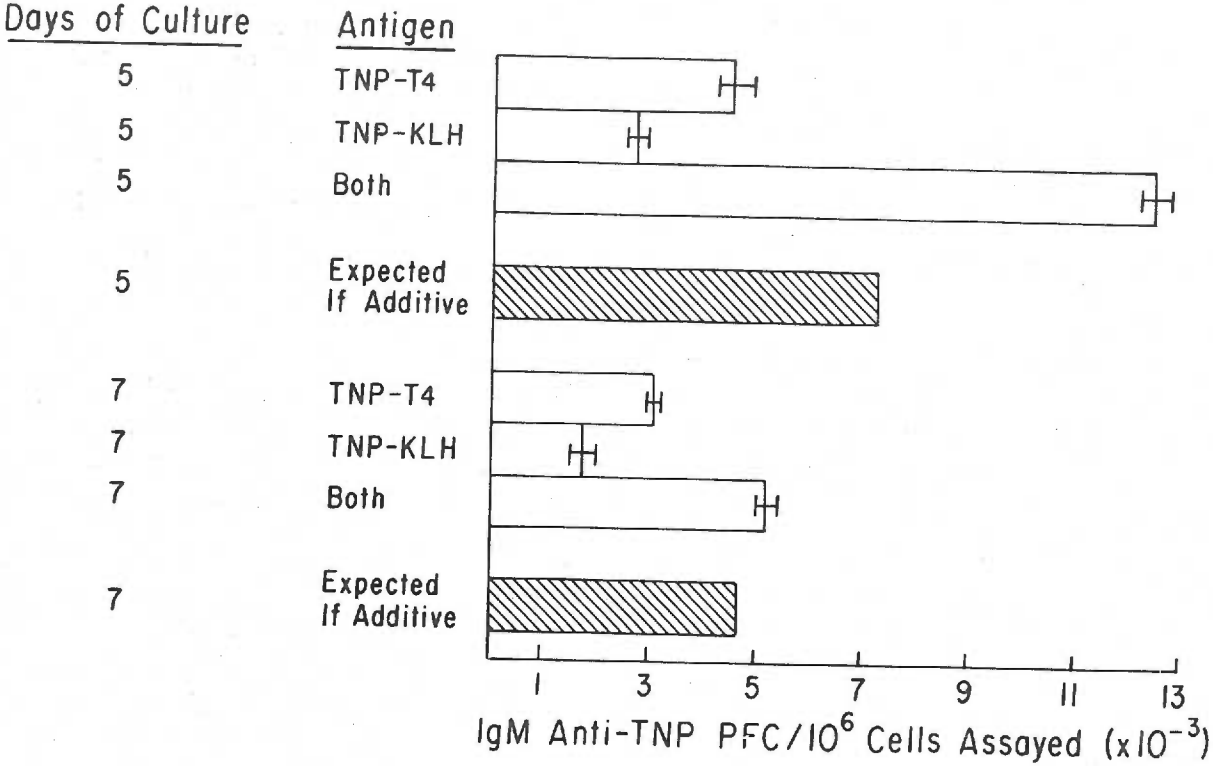

Days of Culture
Antigen
5
TNP-T4
5
TNP-KLH
5
Both
5
Expected If Additive
7
TNP-T4
7
TNP-KLH
7
Both
7
Expected If Additive
1
3
5
7
9
11
13
IgM Anti-TNP PFC/10^6 Cells Assayed (x10^-3)

Figure 2. Addition of secondary IgG anti-TNP PFC responses. Experiment as described in Figure 1 except that IgG anti-TNP PFC were assessed using anti-IgM antibody to suppress direct PFC.

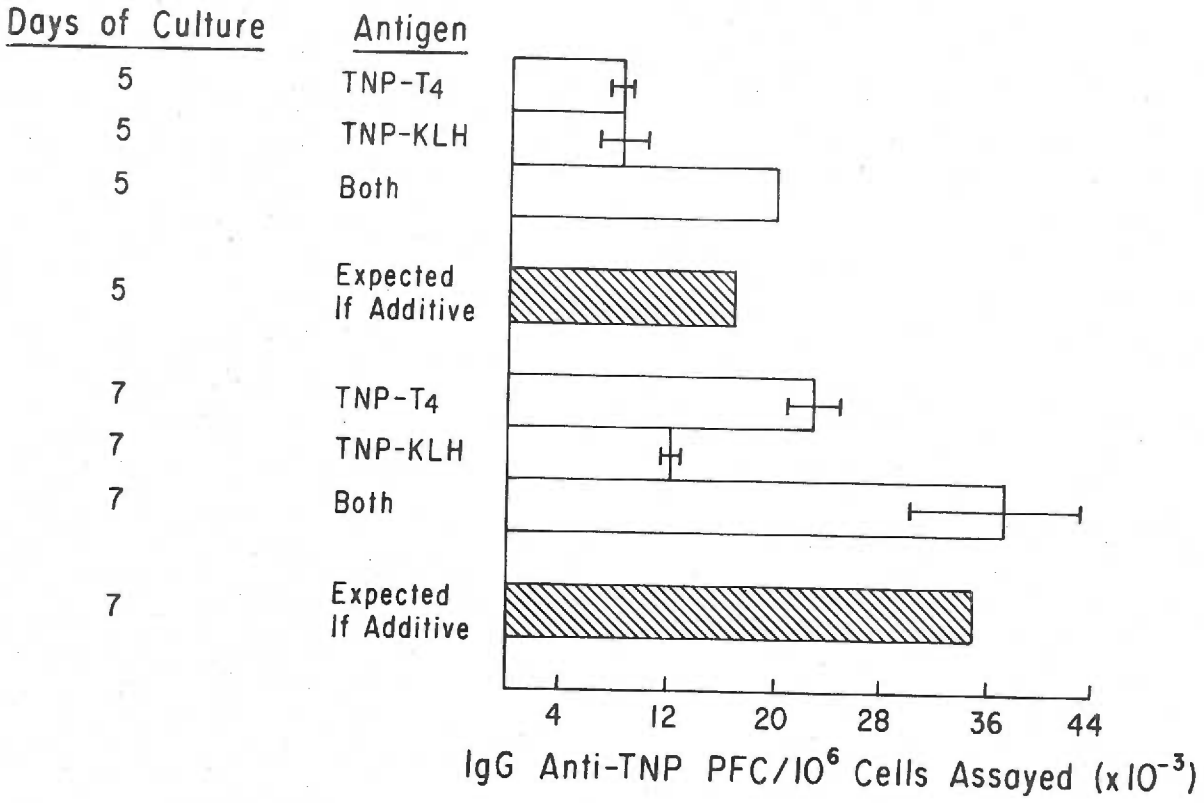
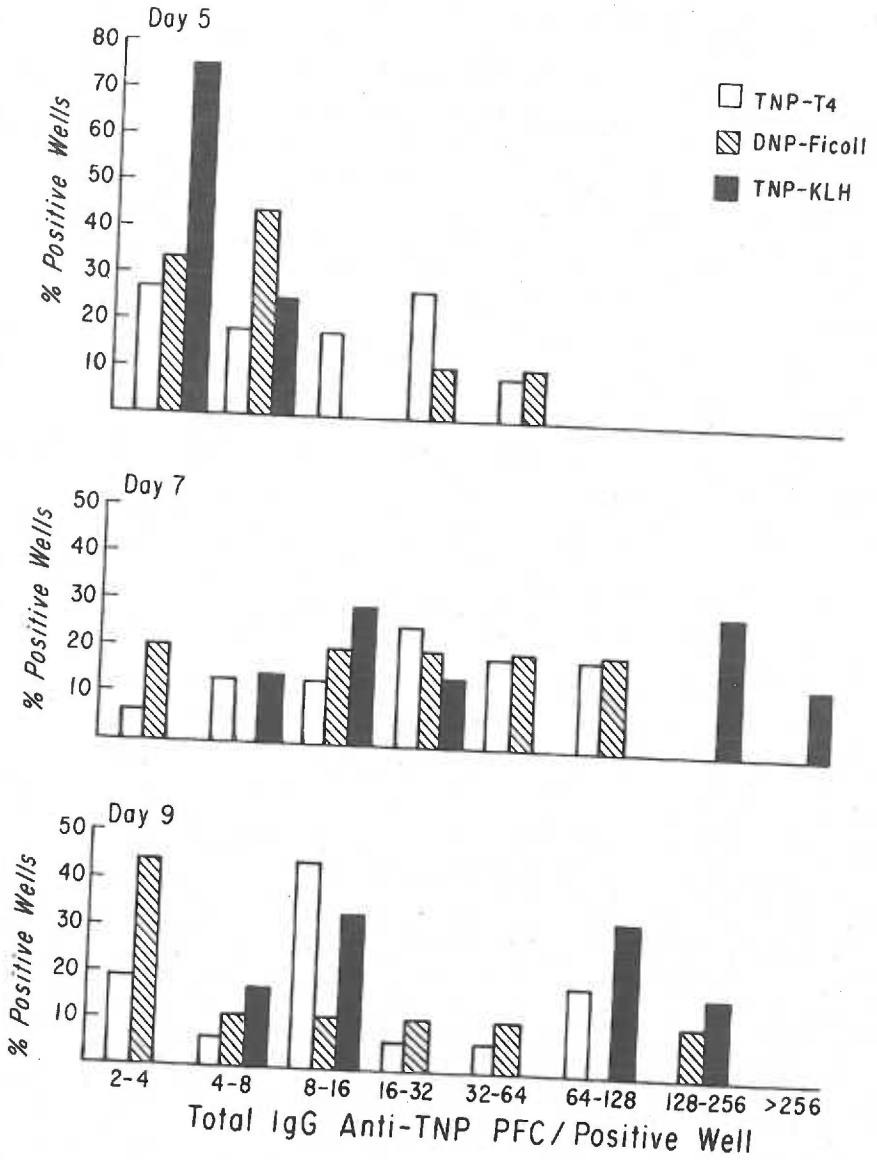

Days of Culture
Antigen
5
TNP-T4
5
TNP-KLH
5
Both
5
Expected If Additive
7
TNP-T4
7
TNP-KLH
7
Both
7
Expected If Additive
4
12
20
28
36
44
IgG Anti-TNP PFC/10^6 Cells Assayed (x10^-3)

Figure 3. In vitro clonal expansion of IgG precursor cells. TNP primed spleen cells were cultured in limiting dilution as in Table I with TNP-T_4, DNP-ficoll or TNP-KLH. Normal thymocytes (3 X 10^4/well) and mitomycin treated KLH primed spleen cells (helper cells) were added as before. The cells were cultured for 5, 7 or 9 days after which the plates were centrifuged and the cells washed in situ. Individual wells were then plaqued against TNP-SRBC and the number of plaques per well determined.


Day 5
% Positive Wells
80
70
60
50
40
30
20
10
TNP-T4
DNP-Ficoll
TNP-KLH
Day 7
% Positive Wells
50
40
30
20
10
Day 9
% Positive Wells
50
40
30
20
10
2-4
4-8
8-16
16-32
32-64
64-128
128-256
>256
Total IgG Anti-TNP PFC / Positive Well

MANUSCRIPT 4

Population Distribution of IgG Memory B Cells

INTRODUCTION

We have reported that based on several criteria there are functionally distinct subpopulations of IgG memory cells (215,223). These cells are distinguished by their responses to so-called thymus independent (TI) and thymus dependent (TD) antigens. These subpopulations termed $B_{1\gamma}$ and $B_{2\gamma}$, respectively, appear to arise from separate memory precursors and thus resemble IgM subpopulations described previously (15,28-35) whose lineage relationships are not understood.

Recently TI antigens have been subdivided into two groups, TI-1 and TI-2 (54,222). The former group consists of antigens such as TNP-lipopolysaccharide (TNP-LPS) and TNP-Brucella abortus (TNP-BA) which are able to induce an IgM response in CBA/N mice (51,222). These mice carry an X-linked B cell defect (207,210), and they do not respond to TI-2 type antigens (54,222) such as TNP-ficoll or DNP-dextran. In addition it appears as though these two groups of TI antigens differ in their susceptibility to anti-δ mediated suppression of primary responses (54) and give additive responses to the hapten when mixtures of TI-1 and TI-2 antigens are added to the same culture (35). Two alternatives have been offered to explain the above findings: 1) TI-1 antigens stimulate an early developing immature B_1 cell (54,222) i.e. a third population of cells or 2) TI -1 antigens for some reason can stimulate a B_2 population but in a thymus independent fashion (35). Here we have utilized the BUdR selective killing method to show that TNP-LPS, a TI-1 antigen can, in fact, stimulate both $B_{1\gamma}$ and $B_{2\gamma}$ memory subpopulations making

unnecessary to invoke a third subpopulation of memory B cell. In addition we show that TNP-T4, a TI antigen used in our earlier studies (215, 223), which was previously unclassified as a TI-1 or TI-2 antigen resembles but is not identical to TI-2 antigens.

MATERIALS AND METHODS

Mice. Adult female Balb/c mice were obtained from Charles River Breeding Labs, Willington, Mass. and were caged in groups of 6 with free access to water and food. (CBA/N x Balb/c)F_1 mice were obtained from our breeding colony. CBA/N parents were the generous gifts of Dr. J. Quintans and Dr. P. Kincade.

Antigens. Trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) was prepared as described previously (57) and had a mole ratio of TNP_{1067}-KLH. TNP-T4 bacteriophage was prepared as described previously (58). Dinitrophenylated-dextran (DNP-dextran) was a gift from Dr. M. Feldman. TNP-LPS was a gift from Dr. D. Morrison. TNP-ficoll was prepared by the method of Blakeslee and Baines (224) using cyanuric chloride to activate TNBS prior to coupling with ficoll.

Immunization. Mice were primed at 2-3 months of age with 3 injections of TNP-KLH-bentonite as previously described (180).

Cell Culture. Spleen cells from at least 3 mice primed 2-4 months previously were pooled and cultured using microtiter plates as previously described (214). The antigen dose is indicated in the results.

Plaque Assay. Anti-TNP plaque-forming-cells (PFC) were detected

using TNP-haptenated sheep red blood cells (TNP-SRBC) as prepared previously (185). Cells from eight replicate microcultures were pooled and plated as one culture for PFC (188). Three such pooled cultures were assayed per experimental point. Cells producing IgG anti-TNP antibody were detected as described previously (185,188,189,205,214).

Selective Suicide. 5 bromouridine deoxyribose (1-5 x 10^{-6} M final concentration, BUdR) was added to cultures 48 hrs after initiation. The cultures were illuminated for 2 hrs on days 3 and 4 as previously reported (223). After the final illumination the cultures were washed and reincubated with fresh culture medium either with or without additional antigen.

RESULTS

Non-Selective Killing of $B_{1\gamma}$ and $B_{2\gamma}$ by BUdR and Light after Initial Stimulation with TNP-LPS. As indicated above TNP-LPS as a TI-1 antigen could stimulate an early developing immature B_1 cell (i.e. a third population of cells) (54,222) or alternatively stimulate a B_2 population in a T independent fashion (35). We felt that BUdR and light treatment could distinguish between these 2 possibilities. If TNP-LPS stimulated a third population of B_γ cells only, the response to TNP-LPS would be suppressed after BUdR killing and the responses to other TI and TD antigens would be unaffected. Alternatively, if TNP-LPS triggered both the $B_{1\gamma}$ and $B_{2\gamma}$ populations, the responses to both TD and TI antigens would be abolished. The results of 1 of 4 such experiments

are shown in Fig. 1. Cells given TNP-LPS prior to BUdR were unable to respond not only to other TI antigens but also to the TD antigen as well, having caused >90% suppression in all cases. Thus, TNP-LPS appears to activate both kinds of B cells and shows no selectivity in the killing observed. This is in complete distinction from our previous BUdR experiments using TNP-T4 (223). The TNP-LPS effect on $B_{2\gamma}$ cells is not due to a nonspecific suppression by LPS since BUdR was required for killing of both populations. Also LPS concentrations up to 100 fold higher than the concentration of TNP-LPS used were ineffective at killing $B_{1\gamma}$ and $B_{2\gamma}$ cells. Since TNP-LPS prior to BUdR eliminates both $B_{1\gamma}$ and $B_{2\gamma}$, it is unnecessary to involve a third population of cells although the possibility of such a population is not eliminated.

$B_{1\gamma}$ and $B_{2\gamma}$ Responses in CBA/N Mice. Quintans has recently reported IgM responses to TNP-T4 in CBA/N mice (225). We have studied the ability of TNP-primed (CBA/N x Balb/c)F_1 female and male mice to express IgG memory responses to TNP-T4 and other TI and TD antigens. In these experiments (CBA/N x Balb/c)F_1 male mice, which express the CBA/N defect, and their normal female littermates were primed with TNP-KLH, rested and their spleens placed into culture with TD, TI-1 or TI-2 antigens. The cultures were assayed on day 7. The results of 1 or 4 such experiments are shown in Fig. 2. The doses of antigen used were optimal in each case (see figure legend). The response to TNP-KLH is severly depressed in male mice ($\geq$ 80%) compared with their female littermates. The responses to TNP-LPS and TNP-T4, although present, are also >80% lower than in the controls. There was no response to TNP-ficoll in male

mice. Thus TNP-T4, since it can stimulate an IgG response in F_1 males, is partly like TNP-LPS but yet TNP-T4 shows a clear difference from TNP-LPS based on BUdR killing data, thus it is not identical to TNP-LPS.

Selective Suicide with TNP-Ficoll. Since mice expressing the CBA/N defect could respond to TNP-T 4 but not to TNP-ficoll and yet TNP-T4 responding cells appeared to be distinct from TNP-KLH responding cells based on BUdR and light experiments (223), it was unclear whether TNP-T4 belonged with the TI-2 antigens. We tried to resolve this question by first challenging primed spleen cells with TNP-ficoll prior to BUdR and light treatment and subsequently rechallenging with TD, TI-1 or TI-2 antigens. Typical results from 1 of 3 experiments are shown in Fig. 3. Note that the responses to both TNP-ficoll and DNP-dextran are inhibited by >90% and yet the response to TNP-KLH is unaffected. Therefore, TNP-ficoll behaves like TNP-T4 with regard to TNP-KLH, both appear to be TI-2 antigens. On the other hand, TNP-ficoll was unable to eliminate completely the TNP-T4 response and the TNP-LPS response was unaffected. Thus TNP-ficoll resembles but is not identical to TNP-T4 and appears distinct from TNP-LPS.

DISCUSSION

Thus far we have obtained evidence for at least two subpopulations of hapten specific memory B cells. Fig. 4 shows a tentative picture for population distribution of antigen reactive IgG memory B cells which can be distinguished readily by BUdR killing data. $B_{2\gamma}$ cells respond

to TD antigen and $B_{1\gamma}$ cells respond to TI antigens such as TNP-ficoll or DNP-dextran. Also, there are three classes of TI antigens: those like TNP-ficoll and DNP-dextran which stimulate only $B_{1\gamma}$ cells, those like TNP-T4 which have a slight overlap into $B_{2\gamma}$ population and those like TNP-LPS which can trigger both $B_{1\gamma}$ and $B_{2\gamma}$. Since T4 is grown in E. coli, it is possible that the overlap into the $B_{2\gamma}$ population seen with TNP-T4 could be due to contamination with TNP-LPS although doses of TNP-LPS lower than 0.01 μg have had no effect on either $B_{1\gamma}$ or $B_{2\gamma}$ in similar BUdR experiments, and as mentioned above doses of LPS up to 10 μg/ml also fail to activate $B_{1\gamma}$ or $B_{2\gamma}$ to BUdR and light sensitivity.

When TNP-ficoll is used as the initial antigen prior to BUdR treatment, the response to TNP-LPS is unaffected (Fig. 3). This was a consistent finding in all experiments where the initial challenge antigen was any TD or TI antigen other than TNP-LPS; the response to TNP-LPS always appeared to be undisturbed. There are at least two possible explanations for this: 1) Since TNP-LPS prior to BUdR and light always kills both $B_{1\gamma}$ and $B_{2\gamma}$ responses, it is possible that elimination of one or the other would allow for a compensatory increased response in the residual population. Such compensation could result through relief from a B cell crowding effect (211). 2) It is possible that TNP-LPS stimulates three subpopulations of memory cells to divide but that only one population continues on to differentiate to antibody secretion. In a preliminary experiment we have challenged primed spleen cells with TNP-KLH and TNP-T4 simultaneously prior to BUdR treatment. In so doing the response to TNP-LPS was lost suggesting that all of the TNP-LPS

responding B cells were triggered to divide by this combination of TI + TD antigens. Although we cannot rule out a third population of memory cells by the present experiments, they indicate at least that TNP-LPS does trigger both $B_{1\gamma}$ and $B_{2\gamma}$ to divide and the necessity to invoke a third subpopulation of TI-1, TNP-LPS responding B cells is not absolutely required. Furthermore, the data suggest that the total number of B memory cell subpopulations responding to the various TNP-antigen forms may be relatively small, perhaps as small as two or three.

Figure 1. Non-selective killing of B_1 and B_2 cells stimulated by TNP-LPS prior to BUdR and light treatment. Cells were given no antigen (controls) or TNP-LPS on day 0 of culture. BUdR (5×10^{-6} M final concentration) was added on day 2 to all cultures including controls and the cells were illuminated 24 and 48 hrs later for 2 hrs on each day. Immediately after illumination the cells were centrifuged, the medium was replaced and the cultures rechallenged with TD or TI antigen as indicated. Antigen doses were optimal: TNP-LPS 90.1 µg/ml), TNP-T4 (6.7×10^3 PFU/ml), DNP-dextran (0.01 µg/ml), TNP-ficoll (0.01 µg/ml), TNP-KLH (0.002 µg/ml). The cultures were assayed 7 days after rechallenge.

EFFECT OF BUdR+LIGHT ON T-LPS STIMULATED IgG PRECURSORS

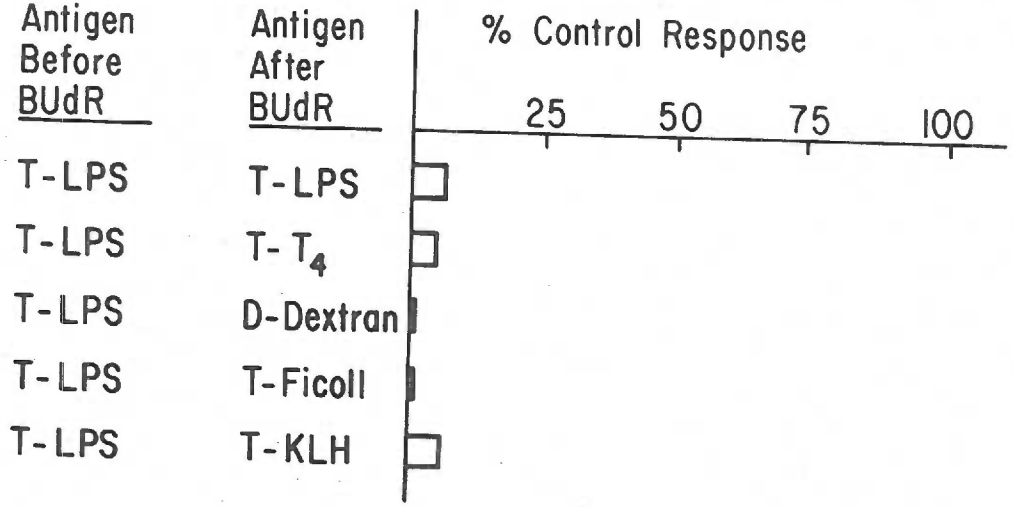


Figure 2. IgG memory responses to TI and TD antigens in (CBA/N x Balb/c) F_1 mice. Spleen cells from primed (CBA/N x Balb/c)F_1 male and female mice were cultured as described in the text with various doses of TD and TI antigen. Only data obtained using the optimum dose for each group are shown although several doses of each antigen were tested in the experiment: TNP-ficoll (0.01 μg/ml), TNP-KLH (0.002 μg/ml, TNP-LPS (1.0 μg/ml), TNP-T4 (6.7×10^3 PFU/ml female; 3.3×10^4 PFU/ml male). The cultures were assayed on day 7.

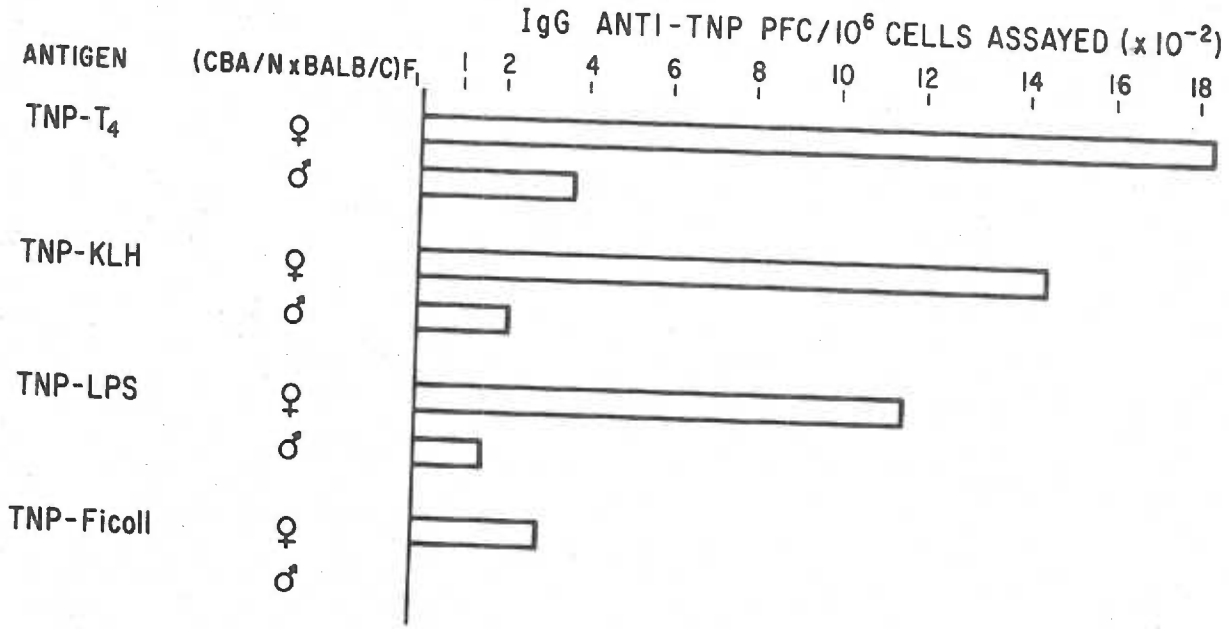

IgG ANTI-TNP PFC/10^6 CELLS ASSAYED (x 10^-2)
ANTIGEN
(CBA/N x BALB/C)F1
1
2
4
6
8
10
12
14
16
18
TNP-T4
♀
♂
TNP-KLH
♀
♂
TNP-LPS
♀
♂
TNP-Ficoll
♀
♂

Figure 3. Selective elimination of $B_{1\gamma}$ cells stimulated by TNP-ficoll prior to BUdR and light treatment. Cells were treated as in Figure 1 except that either no antigen or TNP-ficoll (0.01 µg/ml) was given on day 0.

EFFECT OF BUdR+LIGHT ON T-FICOLL STIMULATED IgG PRECURSORS

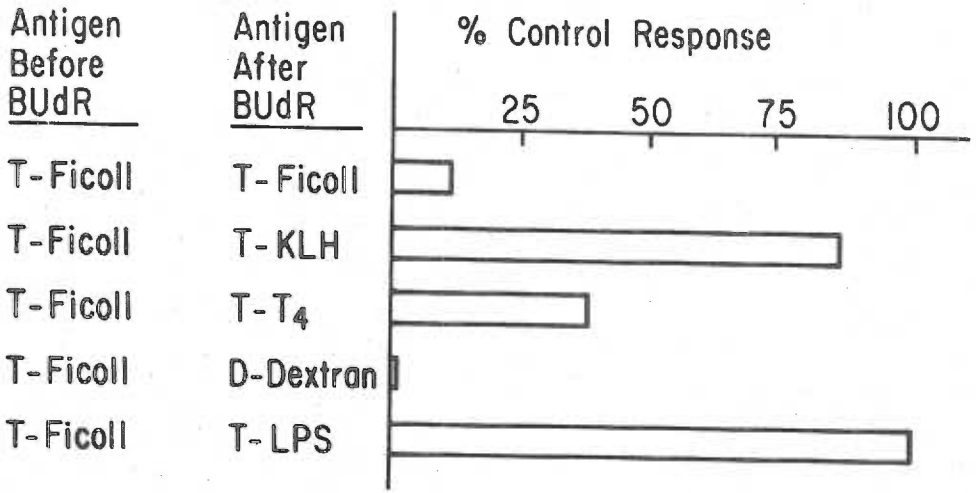
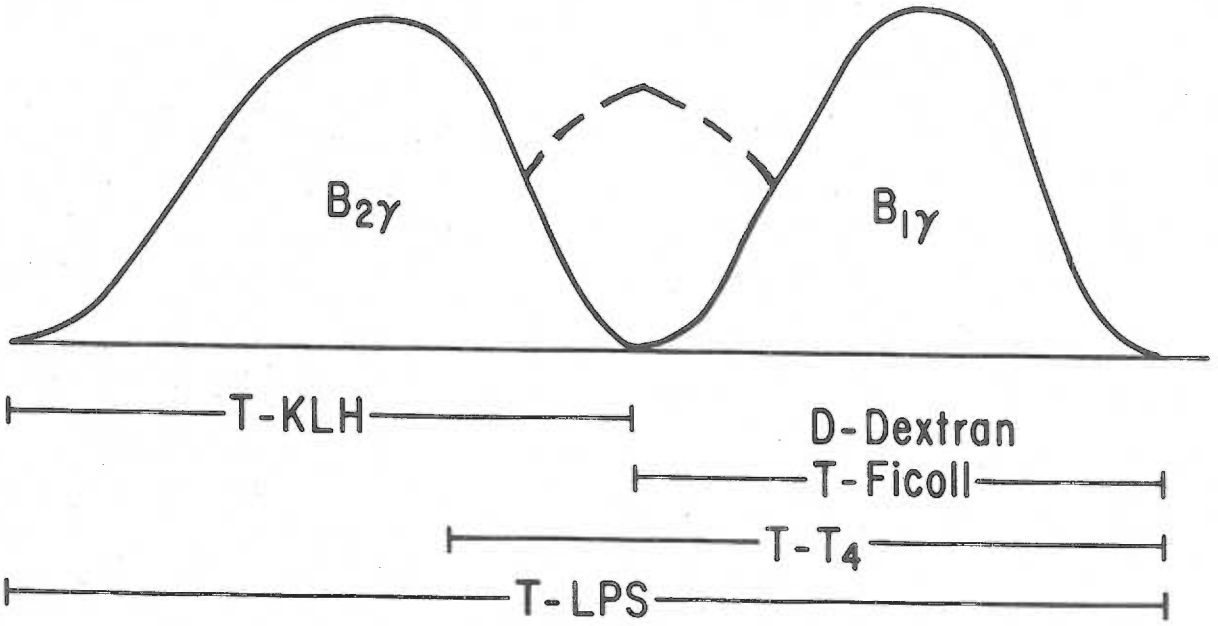


Figure 4. Theoretical population distribution for $B_{1\gamma}$ and $B_{2\gamma}$. Evidence for at least 2 subpopulations of functionally distinct memory B cells responding to the same haptenic determinant has been presented here and elsewhere (215-223). $B_{1\gamma}$ cells respond to TI-2 antigens and to at least one TI-1 antigen (TNP-LPS); $B_{2\gamma}$ cells respond to TD antigens and to at least one TI-1 antigen (TNP-LPS). It is possible that a third population of memory cells exists (dashed line) which would respond only to TI-1 antigens.

Population Distribution of Antigen Reactive IgG Memory B Cells



DISCUSSION

The purpose of this section is to discuss the data presented above collectively, although I shall avoid iteration where possible. I do mean for this to be a compendium of sorts in which I shall stress certain salient features which might not have been dealt with in sufficient detail in the enclosed four manuscripts.

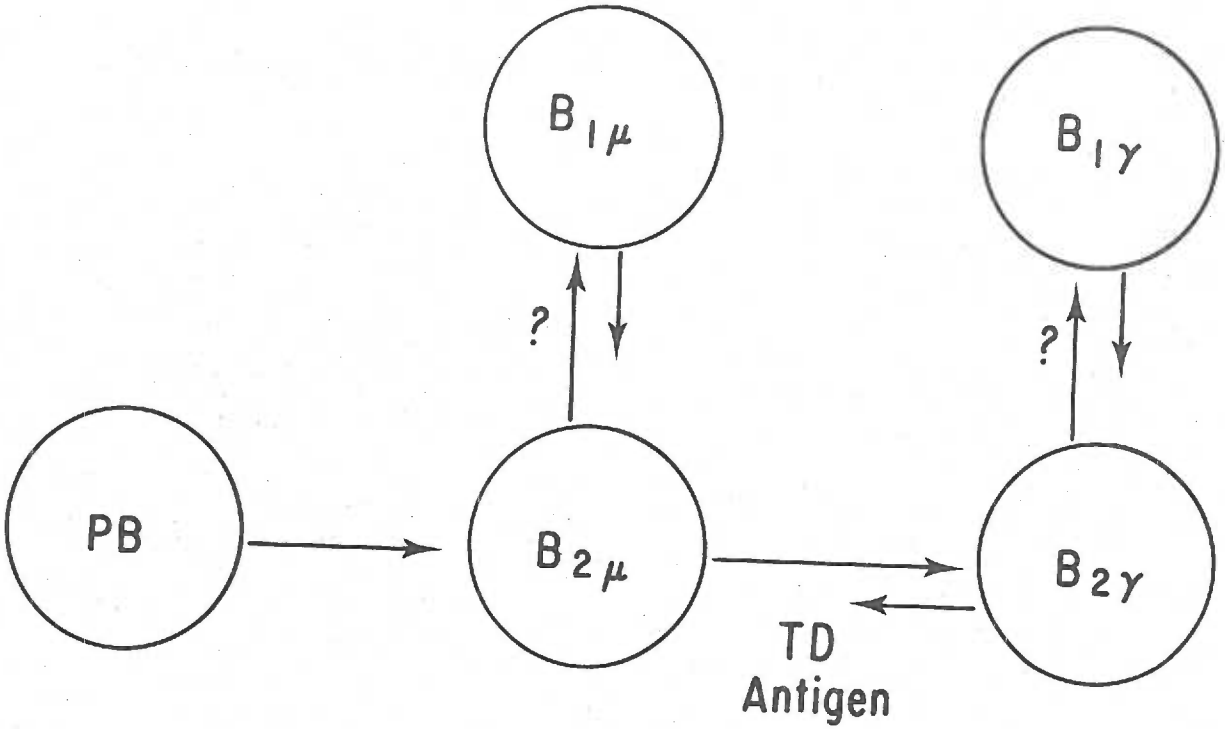
Data presented here and elsewhere (15,28-35) have provided strong evidence for functionally distinct subpopulations of memory and primary B cells. I have proposed the use of the terms $B_{1\mu}$ and $B_{2\mu}$ for TI and TD responding primary B cells and $B_{1\gamma}$ and $B_{2\gamma}$ for TI and TD responding memory B cells, respectively (MSIII). The existance of such subpopulations compels one to re-evaluate one's thinking of the B cell network. There are two examples which speak to this issue. First, in 1966 Claman et al. (226) demonstrated that T cells assisted non-thymus-derived lymphocytes (B cells) to make antibody and by 1970 the concept of cellular cooperativity was firmly established by the elegant studies of Mitchison (178,227,228), who using hapten-carrier conjugates, demonstrated that the ability of B cells to make anti-hapten antibody was preceded by T cell recognition of the carrier. Since TI antigens only stimulated IgM production while TD antigens stimulated IgG and IgA as well as IgM, it was proposed that the B cell responding to TI antigens was a minor population of immature B cells while the TD responding B cell constituted the majority of B cells, both immature and mature and these required T cell help (30). Recent evidence suggests that this is not the case. Precursor frequency analysis of TI and TD responding B

cells shows that $B_{1\mu}$ and $B_{2\mu}$ are present in the spleens of mice with the former generally present in higher numbers (3-10x, 33). Likewise, it appears as though $B_{1\gamma}$ and $B_{2\gamma}$ have similar precursor frequencies (MSIII). Both of these studies were performed using defined haptens (TNP and PC) and there is no reason to expect that the rest of the B cell repertoire should be different. Thus B_1 and B_2 co-exist in apparently equivalent numbers in both primary and secondary B cell compartments. Second, since these subpopulations do exist one should be cautious about interpreting data based on the assumption that all B cells behave alike, i.e. only one population. I have already discussed two instances in which differences between B_1 and B_2 are apparently observed (see above). First the B_1 cells appear to be more susceptible to tolerance induction. One should note that B_1 and B_2 cells could be equally susceptible to tolerance induction. If receptor blockade is the mechanism for the observed tolerance, then the differences observed might be due to B_2 cells being able to break from the tolerant state more easily, presumably with the aid of helper T cells. Regardless of where the difference lies in this system B_1 and B_2 do behave differently. Second, although both B_1 and B_2 cells are present in neonatal animals, only the former can respond to antigenic challenge because of the neonatal suppressor T cell (156). If this suppressor cell acts directly on B cells as suggested by Mosier et al. (229) then the efficiency of the suppression is less with B_1 than with B_2 i.e. they behave differently. However these data await confirmation. Furthermore in the studies reported the suppressor cell could have been acting on helper T cells, thus accounting in part for the apparent difference between B_1 and B_2 responsiveness in newborns; one must say "in part" since there

was evidence that the neonatal suppressors also could interfere with the adult B cell response to DNP-ficoll.

The ontogenic relationships between B_1 and B_2 remain unclear. As mentioned above the two current theories are: 1) independent lineages and 2) $B_2 \rightarrow B_1$. Although I cannot vigourously exclude the independent lineage hypothesis proposed by Kincade (161), I feel the necessity for dual lineages is weak. The reason Kincade proposed such a model is that spleen cells from the CBA/N mouse lack the ability to grow in semi-solid agar i.e. they lack B cell colony-forming-units (CFU). Since CFU appear early in neonatal development he assumed these cells to be immature. However the suppressibility of CFU by anti-μ antibody suggests (162) that these cells are in fact beyond the pre-B state (since pre-B lack surface Ig as determined by immunofluorescence techniques)and are therefore relatively mature. In addition the independent lineage hypothesis does not satisfactorily account for the development of B_1 and B_2 memory cells. If B_1 and B_2 were independent lineages one would have to explain how priming with TI antigens cannot give rise to IgG memory cells whereas priming with TD antigens leads not only to B_2 memory cells but also B_1 memory cells. Although it is possible that the mere binding of a TD antigen to a B_1 cell could cause it to develop into a memory cell, one would have to ask why would a TI antigen which can trigger B_1 cells to divide as well as secrete antibody fail to do the same i.e. generate memory B cells. For these reasons, I favor $B_2 \rightarrow B_1$ and the model shown in Fig. 1 accounts for both primary and memory subpopulations. In addition to the obvious interpretation that $B_2 \rightarrow B_1$ there are several additional points to be gleaned from this figure. The model assumes that the M $\rightarrow$ G switch is valid.

Figure 1 Functional differentiation of primary and memory B lymphocyte precursors.


B1μ
B1γ
?
?
PB
B2μ
B2γ
TD
Antigen

Several laboratories (88,230,231) have presented rather convincing evidence for this assumption. In one of these (231) an individual IgM-bearing cell isolated by micro-manipulation was observed to give rise to IgG secreting daughter cells after mitogenic stimulation. The development of memory $B_{2\gamma}$ cells is shown here to be dependent upon stimulation with a TD antigen. I have purposely left open the question of whether TD antigen alone is sufficient for memory development or whether T cells are also needed. Several laboratories have suggested that memory B cells can develop in the absence of T cells (23-24), but there are singular features in each of these instances which deserve comment. First, the assumption that adult thymectomized, x-irradiated and bone marrow reconstituted (ATxBM) mice are totally devoid of T cells is probably invalid. ATxBM mice has been shown to respond to T cell mitogens (232) and they develop substantial levels of serum IgG and IgA which are absent or severely depressed in athymic nude mice (233). Second memory development could be achieved to SRBC (221) which has a mitogenic component (161) and to the hapten dinitrophenyl (DNP) but only when the hapten-carrier conjugate was injected simultaneously with polymerized flagellin (23) which could also have a mitogenic moiety. Thus although memory developed in athymic nude mice in the absence of T cells a second signal of some sort apparently may have been necessary. By analogy, a second signal presumably from T cells has been suggested for differentiation of B cells to antibody secretion (234,235). It should be mentioned that Braley-Mullen has shown an absolute requirement for T cells during the induction of memory to SIII-RBC (TD) although the expression of memory to SII (TI) did not. I have not tested the relative role of T cells in the development of $B_{1\gamma}$ and $B_{2\gamma}$ in my system but as

in Braley-Mullen's system, I get TI memory expression but not TD memory expression in T cell depleted cultures.

There are two last points concerning the model. First, the nature of signal(s), if any, which induce differentiation from $B_2 \rightarrow B_1$ is not known. Presumably the event would be directed since CBA/N mice lack $B_{1\mu}$ and $B_{1\gamma}$ and not all B_2 cells undergo this differentiative step. However the defect could also be due to blockade of an otherwise naturally occurring second event. A better understanding of the mechanism(s) involved in the hypothesized differentiation scheme might be gained if the step from $B_2 \rightarrow B_1$ proves to be reversible, i.e. cyclical, as indicated by the small arrow in Fig. 1. The fact that B_1 cells are larger cells than are B_2 cells (31) could be taken as suggestive evidence that B_1 cells are perhaps partially activated. If this were so then removal of the non-specific or specific activator substance should result in the activated B_1 cells returning to the resting B_2 stage. Such a scheme for B cell development from small to large to small cell type has been observed by Strober (136,137) as discussed above. In addition the recent report by Bleux et al. (23) that a rare IgG $\rightarrow$ IgM switch may occur accounts for the additional small arrow from $B_{2\gamma} \rightarrow B_{2\mu}$.

Lastly I would like to speculate on the significance of these findings as related to immune responsiveness in humans. The response to bacterial polysaccharide vaccines in man is poor and often leads to hyporesponsiveness (236,237). If these antigens are TI in man as they are in the mouse, then one might predict the difficulty in these instances to be due to the nature of the immunogen. The results of this thesis suggest that a possible means to enhance immunogenicity and memory development would be to couple the immunogen to an appropriate protein

backbone to convert it to a TD antigen. The TD form should be very effective at inducing long-lived memory B cells some of which would be able to respond to the assumed TI form during natural infection. I might add that caution would have to be used in picking a carrier molecule. For example, one could not couple SIII to SRBC as has been done in the murine system. However there is a carrier, diphtheria toxoid, which could be ideally suited for this purpose since most individuals are immunized with this protein early in life.

Finally, I feel B_1 and B_2 cells may play a critical role in the establishment and persistence of certain auto-immune phenomena. One can envision that events which would cause a breakdown in self-tolerance could allow for B_2 cells to respond to self-determinants with the aid of T cells. In addition B_1 cells would develop from the expanding B_2 clones. Once tolerance is broken the normal regulatory controls of the immune system should take over. Thus B_2 cells could be turned off by suppressor T cells affecting helper T cells. In contrast the B_1 cells which are susceptible to T cell regulation but to a lesser degree would continue to secrete anti-self antibody.

I have shown that spleen cells from mice primed with a TD antigen can respond to <u>in vitro</u> challenge with both TI and TD forms of the immunogen. Using this <u>in vitro</u> system I have been able to show that these two antigen forms stimulate distinct memory B cell subpopulations. It is clearly important to understand the relationships between these two subpopulations as such information may provide crucial evidence for our understanding regulation of the humoral immune response and the pathological consequences of its failures.

Finally, I wish to make clear that the classification of antigens

into thymus-dependent and thymus independent categories is artificial and merely reflects molecular conformations which require more or less thymic help in eliciting B cell responses. The data presented above and elsewhere (49,50,52) speak to this point and suggest heterogeneity even among the various TI antigens. No data are as yet available on possible heterogeneity of TD antigens. The ability to distinguish between B cells at different stages of differentiation by using TD and TI antigens may be fortuitous but is nonetheless a convenient and useful means of studying functional differentiation of B lymphocytes.

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