DEFINITION OF CONDITIONS THAT ENABLE ANTIGEN-SPECIFIC ACTIVATION OF THE MAJORITY OF ISOLATED TRINITROPHENOL-BINDING B CELLS*

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Despite decades of study, we still understand relatively little about the role of cell surface immunoglobulin (Ig) in immune activation. Cell surface Ig may act passively by focusing antigen to secondary receptors for mitogenic moieties or antigen-specific T cell factors which may deliver activating signals. Alternatively, cell surface Ig molecules may themselves transduce signals for activation, as is suggested by the ability of anti-Ig antibodies to stimulate thymidine incorporation (1–3).

Recent studies suggest that thymus-dependent initiation of transition of antibody-forming cell precursors from G_0 (in which >95% of peripheral B cells reside [4]) to G_1 is dependent on antigen and major histocompatibility complex $(MHC)^1$ -restricted, antigen-specific T cell help (5, 6). Proliferation and differentiation of G_1 cells into antibody-forming cells is dependent on antigen-nonspecific T cell-derived factors but not antigen. Thus antigen appears to be important only for initiation of transition of G_0 B cells into G_1. Therefore, an improved approach to analysis of antigen-initiated signal generation, transduction, and resultant activation would be to use entry into G_1 as an endpoint rather than thymidine uptake or antibody-forming cell generation, which are dependent on nonspecific accessory functions. This could be achieved by flow cytofluorometric cell cycle analysis of acridine orange-stained cells (4, 7, 8), providing homogeneously antigen responsive B cell populations were available.

A number of investigators have developed methodologies for isolation of antigen-binding cells from normal lymphocyte populations in an effort to produce cell populations homogeneously responsive to antigen. These procedures include the use of fluorescence-activated cell sorting (9), rosetting (10), and solid-phase adsorbents such as haptenated gelatin (11, 12) and nylon fibers (13). Although many cells isolated in this way rebind antigen (11) and some respond to immunogen by thymidine uptake (14), only a small proportion respond to antigen by differentiation into antibody-producing cells (12, 15, 16). This may be due in part to the traumatic treatment used to remove cell-bound antigen after isolation. In an effort to improve upon this methodology, we recently developed a solid-phase cell adsorbent that uses hapten

coupled to gelatin via a cleavable cross-linking reagent (17). Cells isolated using this adsorbent are dissociated under mild reducing conditions not deleterious to cellular function, leaving them associated with only monovalent hapten. As many as 20% of cells prepared in this way respond to immunogen by antibody production.

In this report, we use antigen-binding cells isolated using the method discussed above in conjunction with cell cycle analysis utilizing acridine orange (4, 7, 8) to analyze requirements for activation of trinitrophenol (TNP)-binding murine B lymphocytes. Results in single-cell cultures demonstrate that as many as 48% of TNP-binding cells are activated to enter cell cycle by contact with TNP-*Brucella abortus* (TNP-Ba). This activation is antigen-dependent, -specific, and independent of accessory cells. Activation by TNP-Ba is inhibited by incubation of cells with anti-Fab and anti-μ antibodies but not by anti-δ antibodies. Although these cells rosette with TNP-sheep erythrocytes (SRBC) as well as TNP-Ba, activation of a significant proportion cannot be achieved using immunogenic TNP-SRBC or TNP-ovalbumin (TNP-OVA), even in the presence of interleukin 1 (IL-1), IL-2, B cell growth factor (BCGF), and T cell-replacing factor (TRF) containing supernatants. These data support the notion that MHC-restricted antigen-specific T cell help is required for initiation of G_0 to G_1 transition of antigen-specific B cells by thymus-dependent (TD) antigens (5).

Materials and Methods

Mice. B6D2F1 mice 6–8 wk of age obtained from The Jackson Laboratory (Bar Harbor, ME) were used in these studies.

Antigens. Ba tube antigen was obtained from the National Veterinarian Services Laboratories, Ames, IA. For haptenation, Ba was washed and suspended at 10% packed cell vol/vol in borate-buffered saline (0.035 M boric acid, 0.08 M NaCl, pH 9.4). To 1-ml portions were added various amounts of trinitrobenzene sulfonic acid (J. T. Baker Chemical Co., Philipsburg, NJ) ranging from 0.5 to 10 mg. pH was maintained at 9.4 using 0.2 M NaOH. After 30 min incubation with agitation, TNP-Ba was washed five to six times with Hanks' balanced salt solution (HBSS) and stored at 4°C until use. Unless otherwise stated, TNP_{10}-Ba was used in these experiments. In some cases, TNP-Ba was rhodaminated by incubation at 10% vol/vol in 0.05 M carbonate buffer pH 9.4 containing 100 g/ml rhodamine isothiocyanate for 2 h before washing (TNP-Rho-Ba). Phosphorylcholine Ba was prepared as previously described (17). SRBC were obtained from the Division of Laboratory Animal Resources, Duke Medical Center. Blood from the same sheep was used throughout the study. SRBC were haptenated with trinitrobenzene sulfonic acid as described by Kettman and Dutton (18). OVA and human gamma globulin (HGG) were haptenated using established methods (19) to achieve a haptenation level of TNP_5OVA and TNP_5HGG. TNP-Ficoll with a derivatization level of 40 mol/mol was a gift from Dr. D. W. Scott (Duke University). Lipopolysaccharide (LPS) (0127:B8; lot 674687) was obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY) and dextran sulfate ($DxSO_4$, 500,000 mol wt) was obtained from Sigma Chemical Co., St. Louis, MO.

Antibodies. Rabbit anti-mouse Fab antisera were raised using, as immunogen, Fab fragments of normal mouse IgG prepared using conventional methods (20). Anti-Fab antibodies were affinity purified using normal mouse IgG Sepharose. Mouse IgG was prepared by 18% wt/vol Na_2SO_4 precipitation of normal mouse serum and coupled to Cl Sepharose 4b (Sigma Chemical Co.) using CnBr. Rabbit anti-mouse IgM was raised against affinity-purified hybridoma PAF14 (μ, κ), anti-DNP, a gift from Dr. Robert Giles, University of Mississippi Medical Center, Jackson, MS. This antiserum was made specific by adsorption against TEPC15 (α, κ) and normal mouse IgG Sepharose. Anti-μ antibodies were affinity purified using hybridoma 22.1A4.3 (21) derivatized Sepharose (22.1A4.3 was a gift from Dr. J. Latham Claflin, University of Michigan). Rabbit anti-IgD was raised by immunization with the secreted product of MOPC-1017, a gift from Dr. Fred Finkleman (Uniformed Services University). This antiserum

was made δ chain specific by adsorption on normal mouse IgG Sepharose. The IgG fraction of this serum was isolated using protein A Sepharose. These reagents were determined to be specific by immunofluorescence and by immunoprecipitation of iodinated splenocyte membrane lysates followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (22–24). T cell levels were determined using directly fluoresceinated hybridoma T24 anti-Thy-1 (25). B cell levels were assessed by indirect immunofluorescence using rabbit anti-mouse Fab and fluoresceinated affinity-purified goat anti-rabbit IgG.

Culture conditions.   Splenocytes were depleted of erythrocytes using Gey's solution (26) and in some cases, T cell depleted by hybridoma T24 anti-Thy-1 and complement treatment (25). Cells were cultured in RPMI 1640 (Gibco Laboratories) supplemented with penicillin, streptomycin, glutamine, Na pyruvate, insulin, oxalacetic acid, 2-mercaptoethanol, and 10% fetal calf serum (17, lot 100233; Sterile Systems, Logan, UT). 5×10^2–10^4 cells were cultured per 200-µl capacity microwell. In some cases, single-cell cultures were established in 20-µl volumes of the same medium in Terisaki wells essentially as described by Wetzel and Kettman (27). Cells were plated at a calculated density of 0.5 cells/well. Only wells containing single cells (as determined by visual inspection) were used in single-cell studies of blastogenesis.

Cell Isolation.   The antigen-binding cell isolation procedure used has been described in detail elsewhere (17). This procedure was modified for coupling of TNP to gelatin. To accomplish this, TNP-Lysine (J. T. Baker Chemical Co.) was substituted for PG-glycy-tyrosine in the coupling procedure. Approximately 10^5 TNP-binding cells were isolated per spleen.

Cell Cycle Analysis.   Cell cycle analysis was performed as we have previously described (4, 8) using the Cytofluorograf 50H (Ortho Diagnostic Systems). The acridine orange (AO) staining procedure of Darzynkiewicz et al. (7) was used throughout this study. Briefly, this involves incubation of cells, after mild EDTA-detergent treatment at low pH (0.1% Triton X100, 1mM EDTA, 0.1N HCl) in an aqueous solution of AO (13 µM). Due to the differential binding characteristics of AO to single- and double-stranded nucleic acids, DNA fluoresces green, whereas RNA fluoresces red upon illumination by 488 nm laser light. The cell concentrations in stained samples were adjusted to 1×10^5/ml. Slight variations (<50%) in cell concentration from experiment to experiment had no appreciable effect upon the results as long as the flow rate remained constant. A flow rate of 100 cells/s was generally employed.

For determination of cells in each cycle phase, cytograms of green (DNA) vs. red (RNA) fluorescence were constructed. G_0, G_1, S, and G_2 plus M phase cells were delineated as shown in Fig. 1. The percentage of cells in each cycle phase was determined by integrating each of the gated areas. Such analyses were of 1,000 cells. Demarcations of specific cycle states which are shown were constructed based upon preliminary studies using cell-cycle inhibitors.

To determine the proportion of cells which had entered cycle, 5,000 cell histograms of cell frequency as a function of red fluorescence (RNA content) were constructed and integrated. Those cells displaying greater RNA content than fresh splenocytes (see Fig. 2) were considered to have entered cycle. Inclusion of hydroxyurea (2 mM) (28) to block proliferation in mitogen- or antigen-stimulated cultures lowered the proportion of cells that appeared to have entered cycle by 5–15%. This apparently represents the proportion of cells that underwent cell division in unblocked cultures. Therefore, the assay used may over estimate the proportion of cells which enter cycle by this percentage. In spite of this difference, hydroxyurea was not routinely included in cultures because it significantly reduced the viability of cells cultured for >24 h.

Rosette and Immunofluorescence Assays.   Immunofluorescent staining was assessed using the Cytofluorograf 50H equipped with a 4-W Lexel argon laser. Rosetting was assessed by light and fluorescence microscopy. For TNP-Rho-Ba rosetting, 2×10^4 isolated cells in 200 µl cold HBSS were mixed with 20 µl 0.1% TNP-Rho-Ba in microfuge tubes and spun for 90 s in a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, CA). The pellet was gently resuspended and cells examined by fluorescence microscopy. For TNP-SRBC rosetting, 2×10^4 isolated cells in 200 µl cold HBSS were mixed with 200 µl 1% TNP-SRBC in microfuge tubes and spun for 90 s in a Beckman Microfuge B. After gentle resuspension, rosetting was assessed by light microscopy.

Factors.   Supernatants from WEHI3 (a gift from Dr. Noel Warner, Becton, Dickinson & Co., San Francisco, CA), which were determined to contain IL-1 by conventional assays (29) were used as a source of IL-1. (This preparation was kindly assayed by Dr. Lawrence Lachman,
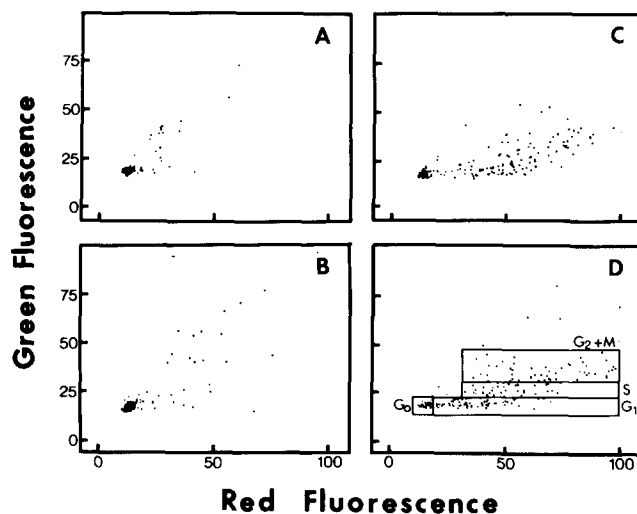
FIG. 1. Cytograms depicting green (DNA) and red (RNA) fluorescence of AO-stained (A) fresh splenocytes (B) TNP-binding cells cultured 48 h in the absence of antigen or (C) stimulated with LPS (50 μg/ml) plus DxSO$_4$ (20 μg/ml) or (D) TNP-Ba (0.01%). Demarcations of cell cycle states were determined in preliminary experiment using cell cycle inhibitors. Each cytogram was constructed using 200 cells.

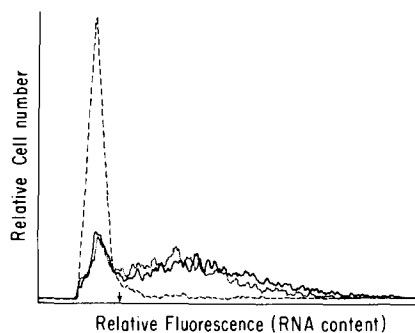Relative Fluorescence (RNA content)

FIG. 2. Histogram depicting the relative red fluorescence (RNA content) of TNP-binding cells cultured 48 h with no antigen (– – –) LPS (50 μg/ml) plus DxSO$_4$ (20 μg/ml) (———) or TNP-Ba (0.01%) (· · · · ·). Those cells exhibiting greater red fluorescence than that channel indicated by the arrow are considered to have entered cycle. Each histogram was constructed using 5,000 cells. This method was used to determine the proportion of cells entering cycle in subsequent experiments. Percent of cells entering cycle in this experiment were 72% for LPS plus DxSO$_4$, 75% for TNP-Ba, and 5% for cultures receiving no antigen.

Immunex, Seattle, WA.) The IL-2- and BCGF-producing EL4 subline (a gift from Dr. Maureen Howard, National Institutes of Health) was cultured with 10 ng/ml phorbol myristic acetate (PMA) as previously described (30). After 48 h, supernatants were harvested and PMA removed by adsorption on activated charcoal and dextran T10 (31). The resultant supernatants were determined to have both IL-2 and BCGF activities (32) and were therefore used as a source of these factors. In the experiments described, WEHI3 and adsorbed EL4 supernatants were used at concentrations at which optimal factor activities were demonstrated. Supernatants of the TRF-producing T cell hybridoma I463.1F2 were assayed for TRF activity in a Mishell and Dutton (33) anti-SRBC response system as previously described (32) and used as a source of TRF at concentrations determined to be optimal.

Results

Phenotype and Antigen-binding Properties of Isolated Cells.   TNP-binding cells from normal mouse spleens were isolated as previously described (17). Briefly, erythrocyte-depleted splenocytes were incubated at 4°C (2 × 10^8 cells/100 mm plate) on disulfide-bonded TNP-gelatin coated plates. Nonbinding cells were removed by washing with cold PBS before binding cells were eluted using 50 mM dithiothreitol and washed with HBSS containing 5% FCS. Cells isolated using this method are not associated with detectable polyvalent hapten or gelatin after isolation (17). Isolated cells were subjected to flow cytometric immunofluorescence analysis to determine the relative proportion of T and B cells within the populations. These studies indicated that 95% of TNP-binding cells were Ig-positive compared with normal splenocytes which were 45% Ig positive. Among the binding cells population, 4% expressed Thy-1 antigen at levels equivalent to 40% of splenocytes and 95% of thymocytes.

We then determined the frequency among isolated cell populations of cells which could rebind TNP-Ba and TNP-SRBC in a rosetting assay. The conjugates formed rosettes with 0–5% of normal mouse splenocytes. In three experiments, frequency of both TNP-Ba and TNP-SRBC binding cells among isolated cells ranged from 70% to 95%. Within single experiments, frequencies of cells binding TNP-Ba and TNP-SRBC were not significantly different.

We next assessed the functional ability of isolated cells to respond to specific antigen and mitogen by entry into cell cycle. Isolated cells were cultured at a density of 10^4 cells/200 μl media alone or in the presence of LPS (50 μg/ml) and DxSO₄ (20 μg/ml) or TNP-Ba (0.01% vol/vol). 12, 24, and 48 h after initiation of cultures, cells were harvested and subject to cell cycle analysis. Whereas entry of some cells <10% into cycle was detected as early as 12 h after initiation of cultures, maximal proportions of cycling cells were always detected at 48 h. Typical 48-h results are shown in Fig. 1. Fresh splenocytes and unstimulated isolated cells were homogeneous with respect to RNA (red fluorescence) and DNA (green fluorescence) content.

This distribution is typical for lymphocytes in G_0 (4). Populations stimulated with LPS and DxSO₄ or TNP-Ba include cells containing increased levels of RNA, indicative of entry into G_1, and some cells with increased levels of DNA indicating entry of cells into S, G_2, and M. To quantitate the proportion of cells that have entered cycle, histograms relating cell frequency to RNA content (red fluorescence) were constructed (Fig. 2). Those cells containing elevated red fluorescence intensity to the right of the channel marker were considered to have entered cycle. In this experiment, 5% of isolated cells entered cycle under nonstimulatory conditions, whereas 72% and and 75% entered cycle in response to LPS, DxSO₄, and TNP-Ba, respectively.

Specificity of Activation with TNP-Ba.   In view of the surprising efficiency of TNP-Ba in inducing activation, we thought it important to determine whether Ba could be acting as a polyclonal activator in this system. Therefore, we examined antigen specificity of TNP-binding cell responses to TNP-Ba. T cell-depleted normal spleno-cytes and TNP-binding splenocyte populations were cultured (10^4/200 μl culture) with no added antigen, in the presence of underivatized Ba or with various TNP-Ba conjugates (0.01% vol/vol) prepared as described in Materials and Methods. As shown in Fig. 3, conjugates prepared using 1–10 mg TNBS/ml of 10% vol/vol Ba caused activation of 49–61% of antigen-binding cells. Over the course of 10–12 such experi-
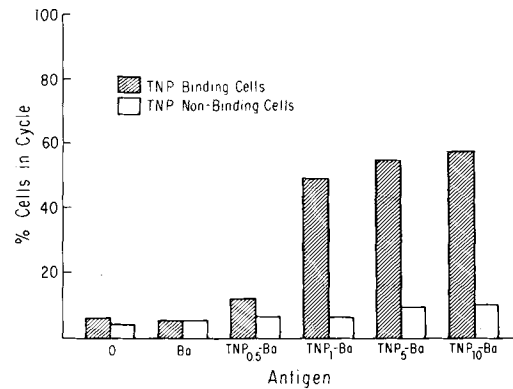
Fig. 3. Histogram depicting the frequency of T cell-depleted splenocyte and TNP-binding cells that have entered cycle 48 h after exposure to no antigen (0), underivatized Ba, and TNP-Ba conjugates prepared using 0.5, 1, 5, or 10 mg TNBS/ml 10% vol/vol organisms. The proportion of cells in cycle was determined by AO staining and analysis of 5,000 cells/culture.
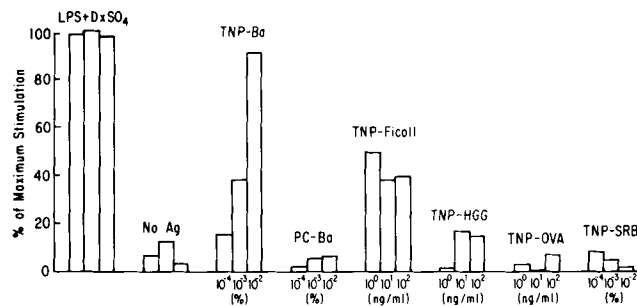

Fig. 4. Histogram demonstrating the frequency of TNP-binding cells that have entered cycle 48 h after exposure to LPS (50 μg/ml) plus DxSO$_4$ (20 μg/ml, triplicate cultures), no antigen (triplicate cultures shown), and various doses of TNP-Ba, PC-Ba, TNP-Ficoll, TNP-HGG, TNP-OVA, or TNP-SRBC. Maximum stimulation achieved using LPS plus DxSO$_4$ was 62% of cells entering cycle. Proportion of cells in cycle was determined by acridine orange staining and analysis of 5,000 cells/culture.

ments, the percent of antigen-binding cells activated by these conjugates has ranged from 45 to 90%. However, percentages activated in replicate cultures in single experiments are always within 5% of each other but vary more significantly between experiments. 4–8% of cells from both populations were activated when co-cultured with no antigen or underivatized Ba. It appears that higher conjugates of TNP-Ba activate a low but significant (8–10%) proportion of normal B cells.

Activation of Antigen-binding Cells by Other TNP Immunogens. We next assessed the ability of another thymus-independent (TI) immunogen, TNP-Ficoll, and TD immunogens TNP-HGG, TNP-OVA, and TNP-SRBC to activate TNP-binding cells under these conditions. In these experiments, antigens were added at varying concentrations over three log increments, which in all cases included doses optimally immunogenic for induction of antibody production in vitro. As shown in Fig. 4, maximal antigen-specific activation was achieved using 0.01% TNP$_{10}$Ba, which activated 95% of the number of cells activated in triplicate cultures by LPS (50 μg/ml) plus DxSO$_4$ (20 μg/ml). A significant but smaller proportion of cells were also

activated by TNP-Ficoll. The optimal doses of these immunogens determined in this system, 0.01% for TNP-Ba and 1 ng/ml for TNP-Ficoll, were also optimally immunogenic for generation of antibody responses in vitro (data not shown). Significant activation did not occur in cultures receiving neither antigen nor the TD antigens TNP-HGG, TNP-OVA, or TNP-SRBC at any dose. These results suggest that T cell help may be required for initiation of G_0 to G_1 transition by thymus-dependent antigens. This has also been suggested by studies of Andersson et al. (6) indicating that antigen-specific Ia-restricted help is required for blastogenesis in response to TD erythrocyte antigens. In view of the fact that nonspecific helper factors effectively replace T cells in in vitro antibody responses to SRBC, we examined their ability to facilitate G_0 to G_1 transition of TNP-binding cells initiated by TNP-SRBC. IL-1, IL-2, BCGF, and TRF were added with TNP-SRBC to cultures in varied permutations at doses previously determined to be optimal in conventional assays (see Materials and Methods). Activity of the factor preparations has been defined in detail elsewhere (32). As shown in Fig. 5, whereas 79% of cells were activated by TNP-Ba and only 8% by PC-Ba, no combination of 0.01% TNP-SRBC and nonspecific factors caused significant activation. Although a slightly increased frequency (12%) of cells were activated in cultures receiving antigen plus IL-1 and IL-2, this increase is probably not significant. These data suggest that induction of antibody formation by B cells using SRBC antigen and nonspecific factors in vitro may be a result of stimulation of a small "preactivated cell" population. This possibility is consistent with observations that soluble protein antigens plus nonspecific factors are effective stimulants of recently primed but not unprimed B cells (34). They further support the notion that TD activation of G_0 B cells may require antigen-specific, MHC-restricted T cell help as discussed above.

Role of Receptor IgM and IgD in Activation by TNP-Ba. We undertook studies of the role of receptor Igs in TI activation for two reasons. We wanted to be sure that activation by TNP-Ba was mediated by surface Ig binding and not some nonspecific mechanism. In addition, we wanted to determine whether TI activation in this system requires both sIgM and sIgD or only requires sIgM as has been shown for in vitro antibody responses to TNP-Ba (35). Antigen-binding cells were cultured with 0.01% TNP-Ba alone or with rabbit antibodies against mouse Fab fragments, μ, or δ chains used at concentrations optimal for capping of surface Ig. Equivalent concentrations of normal rabbit Ig was used as a control. After 48 h, cells were harvested and subjected to cell cycle analysis. Results of two experiments are shown in Fig. 6. Whereas 50–60% of cells were activated in the presence of normal rabbit Ig, anti-Fab and anti-μ antibodies reduced the frequency of cells activated to near background levels. Anti-δ chain antibodies had no inhibitory effect, suggesting that expression of this isotype is not required for activation by this TI antigen. These data suggest that G_0 to G_1 transition follows from interaction of TNP-Ba with IgM but not IgD.

Accessory Cell Dependence of TNP-Ba-mediated Activation. The ability of TNP-Ba to activate TNP-binding cell populations containing $\geq 96\%$ Ig-positive cells suggests that TNP-Ba may stimulate activation and proliferation in the absence of accessory cell function. We undertook two approaches to determine the accessory cell dependence of B cell activation by TNP-Ba. In the first, TNP-binding cells were cultured at varied concentrations ranging from 1.25×10^3 to 10^4 cells per 200-μl culture with 0.01% TNP-Ba, and activation was assessed 48 h later by cell cycle analysis. At densities
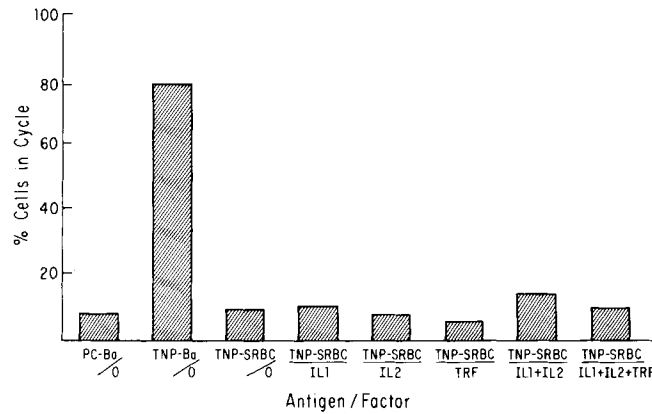
FIG. 5. Histogram demonstrating the inability of the nonspecific factors IL-1 (from WEHI3), IL-2 + BCGF (from EL4), and TRF (from I4G3) either alone or mixed to facilitate entry of TNP-binding cells into cycle in response to TNP-SRBC. TNP-binding cells were cultured 48 h in the presence of PC-Ba, TNP-Ba, or TNP-SRBC plus factors before AO staining and analysis of 5,000 cells/culture.
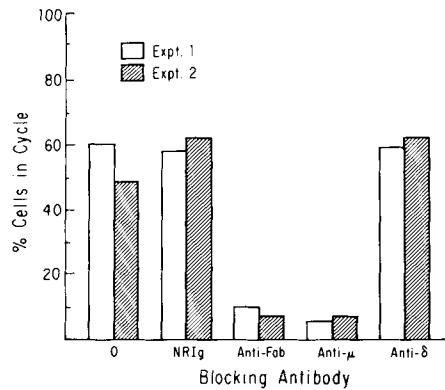

FIG. 6. Histogram demonstrating the inhibitory effects of NRIG, Anti-Fab, Anti-μ, and Anti-δ on TNP-Ba induced entry of TNP-binding cells into cycle. TNP-binding cells were cultured 3 h in medium alone or in the presence of optimal capping concentrations of affinity-purified antibodies (250 μg/ml) or 50 μg/ml normal rabbit immunoglobulin before TNP-Ba was added. After 48 h cultures were harvested and stained with AO. 5,000 cells were analyzed per culture.

below 5×10^3 per cultures, cells are sparsely scattered so that cell-cell contact is rarely seen upon microscopic examination. If antigen presentation or cell-cell contact were necessary for TNP-Ba-mediated activation, activation should be relatively inefficient in these low density cultures. Fig. 7 shows the results of such an experiment. Frequency of cells activated remained constant as cell density decreased, suggesting that accessory function is unnecessary for activation by TNP-Ba. In a second approach, single-cell cultures of antigen-binding cells were prepared in Terisaki trays as described by Wetzel and Kettman (27), except that TNP-Ba (0.01%) was used instead of mitogen stimulation. For each population, 192 wells were plated at 0.5 cells/well. Wells containing single cells were identified and scored for cell division at 24, 48, and 72 h. Cell division occured in only 4% of cultures receiving no stimulus and in 81% of wells receiving LPS and DxSO4. This is in aggrement with studies of Wetzel and Kettman
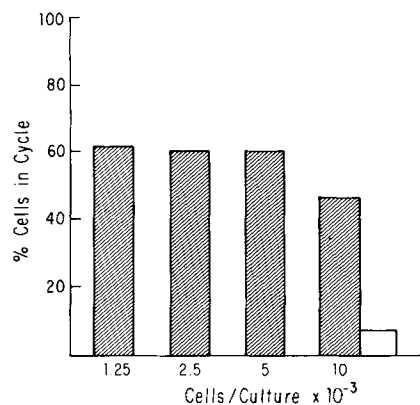
Fig. 7.   Histogram demonstrating the effect of cell density on the proportion of TNP-binding cells that enter cycle within 48 h of stimulation with TNP-Ba (0.01%). Cells were cultured at 1.25-10 × 10^3 cells per well in flat-bottomed 96-well microtiter plates with antigen for 48 h before being stained with AO. 5,000 cells were analyzed per culture. T cell-depleted splenocytes stimulated with TNP-Ba were included as a negative control.

(27), who found that ~80% of single B cells could be activated in these cultures with LPS + $DxSO_4$. Finally, cell division occurred in 48% of wells receiving TNP-Ba. These results indicate that TNP-Ba is autonomous, requiring no accessory cell function for activation of a large proportion of TNP-binding cells.

Discussion

We have assessed the ability of a number of TD and TI TNP conjugates to stimulate isolated TNP-binding B cells to leave the G_0, or the quiescent state, and enter cell cycle. We found that up to 70% of these isolated cells were activated by TNP-Ba, whereas a lower but significant proportion were activated by TNP-Ficoll. Activation by TNP-Ba was found to be antigen specific and independent of accessory cells, occurring with approximately equal efficiency in bulk and single-cell cultures of antigen-binding cells. No activation was achieved using TD conjugates of TNP-HGG, TNP-OVA, and TNP-SRBC.

Of critical importance in the interpretation of these studies is the validity of entry into cell cycle as a criterion for lymphocyte activation. Whereas G_0 to G_1 transition is by definition activation, it could be argued that it bears little relevance to generation of an antibody response. The importance of G_0 to G_1 transition of B cells making an immune response is supported by direct and indirect evidence. Although it has been demonstrated that 95% of fresh splenic B cells are in G_0 (4, 8), populations exposed for 48 h to mitogenic levels of LPS or anti-mouse Fab antibodies contain predominantly G_1 cells with some S, G_2, and M phase cells (J. Monroe and J. Cambier, manuscript in preparation). Entry of lymphocytes into G_1 is correlated with increase in cell diameter from 4.5-5.5 μm to 6-10 μm (36), which is correlated with a decrease in cell density. A similar transition was observed in early studies by Haskill (37), demonstrating that within 10 h of antigen stimulation in vivo, anti-SRBC antibody-forming cell precursors undergo a decrease in density. Taken together, we interpret these studies as indicating that within 10 h of exposure to antigen in vivo, antibody-forming cell precursors may enter cycle. Also in support of the importance of G_0 to G_1 transition in immune responses is the comparative ultrastructure of lymphocytes and

antibody-secreting cells. The latter possess a well-developed secretory apparatus and polyribosomes for high rate production of antibody. As a result of their increased ribosomal RNA content, these cells should exhibit a G_1 AO staining pattern. We have examined this question directly by sorting 96-h LPS- and $DxSO_4$-stimulated B cell populations containing Ig-secreting cells on the basis of size to obtain subpopulations restricted in cycle phase distrubution (8, and J. Cambier and W. Havran, manuscript in preparation). Frequencies of Ig-secreting cells among these subpopulations were then determined using the reverse plaque-forming cell assay. Results indicate that Ig-secreting cells are entirely restricted to the G_{1B} population. This indicates that in the strictest sense, antibody-forming cell precursors must enter G_1 to become antibody-secreting cells. This does not mean, however, that all cells in G_1 are destined to secrete antibody, i.e., G_1 does not represent a commitment to antibody secretion. Most G_1 cells are presumably destined to proliferate before becoming memory cells or differentiating to become antibody-secreting cells. Unfortunately, AO cell cycle analysis does not allow quantitation of the proportion of cells that enter S during the culture period. This is due in large part to the fact that the duration of the S phase is normally only a few hours. The replicative potential of these cells is much better reflected in the single-cell culture studies, which indicate that by 72 h after stimulation by TNP-Ba, as many as 48% of TNP-specific cells undergo cell division. This suggests that most cells that enter cycle in response to this antigen have the potential to replicate.

It is perhaps surprising that TNP-Ba causes activation of such a large proportion of antigen-binding cells. The ineffectiveness of underivatized Ba and phosphorylcholine-coupled Ba in activation of unfractionated or TNP-binding cells argues against it being a polyclonal activator. It is unlikely that higher doses of these bacteria would be mitogenic because at the doses used (0.01%) bacteria are confluent on the bottom of microwells. Therefore, repeated bacterial contact with all cells is virtually assured. It should be noted, however, that TNP-Ba prepared with 25 mg TNBS/ml of 10% bacteria stimulate entry of a significant proportion (10–20%) of normal B cells into cell cycle (data not shown). The nature and specificity of this stimulation is unclear. In studies described here, we have intentionally used conjugates which gave only specific and cell surface Ig-dependent activation.

The observation that the majority of TNP-binding B cells are activated by TNP-Ba is unexpected in part because of voluminous literature indicating the existence of several subsets of B cells which differ in carrier responsiveness. For example, TI and TD antigen-responsive B cells differ in size (38), tolerance susceptibility (39–41), LyB antigen expression (42), inhibitability by anti-isotypic antibodies, and frequency (35, 41, 43, 44). TI responsive populations may be further separated into TI_1 and TI_2 antigen-responsive subsets on the basis of tolerance susceptibility (39, 41), LyB5 expression (45), requirements for IgD in activation (46), and distribution in CBA/N mice (47). Studies comparing the frequency of cells responsive to these antigens and the magnitude of bulk culture responses to them have generally indicated that TD antigen-responsive cells are less frequent than TI antigen-responsive cells. However, our own studies using limiting dilution analysis to compare frequencies of B cells responsive to TNP-SRBC (TD) and TNP-Ba (TI_1) antigens used in this study indicate that among $B_6D_2F_1$ splenocytes, these cells are present at approximately equal frequencies (J. Cambier, unpublished observation). In view of these findings, evidence that the TI_2 antigens such as TNP-Ficoll might activate an additional B cell

subpopulation, and the strong possibility that still other B cell subpopulations might well exist, we did not expect that TNP-Ba would activate 80% of TNP-binding cells, as seen here. A hypothesis to explain these findings may be proposed based upon our studies and those by Anderson et al. (48) and Wetzel and Kettman (27), which indicate that although as many as 80% of cells are activated to proliferate by mitogens, only one-third of them differentiate to antibody production. Similarly, we have previously demonstrated that at most only about one-fifth of hapten-binding cells isolated using this method (one-third the number that enter G_1) respond in limiting dilution analysis by production of anti-hapten antibody (17). It is possible that B cell subsets defined by antibody production are not reflected at the level of G_0-G_1 transition. For example, whereas TNP-Ba stimulates G_0 to G_1 transition of most TNP-binding B cells, it may only stimulate antibody production by a subset definable on the basis of size, tolerance susceptibility, etc. A Ba-specific event unrelated to G_0 to G_1 transition would determine the lymphocytes destined to become antibody-forming cells. This has been suggested by Scott and Alexander (49) based upon their observations and those of Mosier (50) of the ability of various fluorescein (FL) immunogens to cross prime for subsequent responses to one another. These studies, which used BUdR and light suicide, indicate that FL immunogens such as FL-Ficoll stimulate proliferation of B cells responsive to subsequent challenge by FL-POL, FL-LPS, FL-PPD, FL-Ficoll, FL-Ba, and FL-HRBC. In their studies, unlike ours, hapten erythrocyte complexes also stimulated all B cell subsets. This is easily explained by the fact that in their studies, carrier-specific T cells were not eliminated before cross priming. Significant numbers of T cells are not present among isolated antigen-binding cells. Given these observations, it is surprising that TNP-Ficoll did not activate a proportion of B cells similar to that activated by TNP-Ba, even though cells activated by TNP-Ficoll to produce antibody (LyB5$^+$) are a subset of TNP-Ba antibody-producing cell precursors (LyB5$^+$ and LyB5$^-$) (47). Numerous possibilities may explain this difference, e.g., limiting accessory cells may be necessary for TNP-Ficoll-mediated activation (42).

Activation of TNP-binding B cells at low cell density and in single-cell cultures demonstrates the independence of TNP-Ba on any accessory cell function. Similar autonomy of FL-POL in activating a proportion (2–3%) of isolated fluorescein-binding cells to proliferation and antibody secretion in single-cell cultures has recently been reported (16). The very low frequency of responsive cells observed relative to those we report here may be a function of the different immunogens used or the differences in methods utilized to isolate cells.

Particularly noteworthy in these studies is the total inability of the TD TNP immunogens TNP-SRBC, TNP-HGG, and TNP-OVA to initiate G_0 to G_1 transition of these cells. This is in spite of the fact that the cells bind TNP-SRBC avidly. Activation could not be facilitated by further addition of optimal doses of IL-1, IL-2, BCGF, and TRF-containing supernatants to cultures. The most obvious interpretation of these data is that antigen-specific helper T cells or antigen-specific T cell factors are essential for initiation of G_0 to G_1 transition by TD antigens. Such a requirement has recently been demonstrated by Andersson et al. (6, 51). These workers have shown that blastogenesis of quiescent cells in response to horse erythrocytes requires Ia-restricted, antigen-specific T cell help. This activity occurs in supernatants of helper T cell lines in two fractions of 125,000 and 55,000 mol wt. Failure to activate cells by

exposure to polyvalent TD antigens indicates that receptor cross-linking is insufficient to generate G_0 to G_1 transition of B cells. This appears inconsistent with results of studies using anti-Ig antibodies for activation of B cells, which suggest that proliferation in response to these agents is thymus independent (3, 52). However, more recent studies in which T cells were rigorously eliminated suggest that T cells are necessary for this response.[2] Thus, our data indicate that T cell help may be necessary for generation of very early activation events, including the increased RNA production that is indicative of entry into G_1. Our further studies in this area will address the precise roles of receptor-ligand binding and specific T cell help in generating the G_0 to G_1 transition.

Summary

In an effort to further elucidate the early cellular events in generation of antibody responses, we have determined the requirements for antigen-specific initiation of the G_0 to G_1 transition by isolated trinitrophenol (TNP) -binding B lymphocytes. TNP-binding cells were isolated from normal $B6D2F_1$ splenocyte populations using hapten affinity fractionation on disulfide-bonded TNP-gelatin-coated plates. Populations prepared in this way are $\geq96\%$ immunoglobulin positive and 70–95% antigen binding. Isolated cells were cultured for 48 h in the presence of a variety of TNP conjugates including TNP-*Brucella abortus* (Ba), TNP-Ficoll, TNP-sheep erythrocytes (SRBC), TNP-human gamma globulin (HGG), or TNP-ovalbumin (OVA) before being harvested and subjected to acridine orange cell cycle analysis. As many as 80% of cells were in cycle by 48 h in response to TNP-Ba, a thymus-independent (TI$_1$ antigen. A smaller proportion ($\simeq40\%$) were in cycle in response to TNP-Ficoll, a TI2 antigen. Significant activation was not detected in cultures challenged with the thymus-dependent immunogens TNP-SRBC, TNP-HGG, and TNP-OVA. Addition of interleukin 1 (IL-1), IL-2, B cell growth factor, and/or T cell-replacing factor to cultures did not facilitate responses to these immunogens, suggesting a requirement for antigen-specific T cell help for entry into cell cycle induced by thymus dependent antigens. Activation by TNP-Ba was antigen specific and independent of accessory cells, occurring with equal efficiency in bulk and single-cell cultures. Activation by TNP-Ba was inhibitable by anti-Fab and anti-μ antibodies, but not by anti-δ antibodies.

Results indicate that activation of TNP-binding cells to enter cell cycle by TNP-Ba is independent of accessory cells and requires interaction of antigen with cell surface IgM. Exposure to thymus-dependent TNP-immunogens plus nonspecific helper factors is insufficient to cause entry of TNP-binding cells into cycle.

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[2] M. Howard, B. Johnson, and W. Paul. Accessory cell-derived factors required for anti-Ig-induced B cell proliferation. Manuscript submitted for publication.

References

1. Sell, S., and P. G. H. Gell. 1965. Studies on rabbit lymphocytes *in vitro*. I. Stimulation of blast transformation with an anti-allotype serum. *J. Exp. Med.* **122:**423.

2. Sell, S., D. S. Rowe, and P. G. H. Gell. 1965. Studies on rabbit lymphocytes *in vitro*. III. Protein, RNA, and DNA synthesis by lymphocyte cultures after stimulation with phytohaemagglutinin, with staphylococcal filtrate, with anti-allotype serum, and with heterologous antiserum to whole rabbit serum. *J. Exp. Med.* **122:**823.

3. Parker, D. C. 1975. Stimulation of mouse lymphocytes by insoluble anti-mouse immunoglobulin. *Nature (Lond.).* **258:**361.

4. Monroe, J. G., and J. C. Cambier. 1982. Cell cycle dependence for expression of membrane associated IgD, IgM and Ia antigen on mitogen-stimulated murine B lymphocytes. *Ann. N. Y. Acad. Sci.* **399:**238.

5. Melchers, F., J. Andersson, W. Lernhardt, and M. Schrier. 1980. Roles of surface-bound immunoglobulin molecules in regulating the replication and maturation to immunoglobulin secretion of B lymphocytes. *Immunol Rev.* **52:**89.

6. Andersson, J., M. H. Schreier, and F. Melchers. 1980. T cell-dependent B-cell stimulation is H-2 restricted and antigen dependent only at the resting B-cell level. *Proc. Natl. Acad. Sci. U. S. A.* **77:**1612.

7. Darzynkiewicz, Z., T. Sharpless, L. Staiano-Coico, and M. R. Melamed. 1980. Subcompartments of the G_1 phase of cell cycle detected by flow cytometry. *Proc. Natl. Acad. Sci. U. S. A.* **77:**6696.

8. Monroe, J. G., W. L. Havran, and J. C. Cambier. 1982. Enrichment of viable lymphocyte in defined cycle phases by sorting on the basis of pulse width of axial light extinction. *Cytometry.* **3:**24.

9. Julius, M. H., T. Masuda, and L. A. Herzenberg. 1972. Demonstration that antigen-binding cells are precursors of antibody-producing cells after purification with a FACS. *Proc. Natl. Acad. Sci. U. S. A.* **69:**1934.

10. Bach, J. F., J. Y. Mullen, and M. Dardenne. 1970. In vivo specific antigen recognition by rosette forming cells. *Nature (Lond.).* **227:**1251.

11. Haas, W., and J. E. Layton. 1975. Separation of antigen-specific lymphocytes. I. Enrichment of antigen-binding cells. *J. Exp. Med.* **141:**1004.

12. Scott, D. W., M. Venkataraman, and J. J. Jandinski. 1979. Multiple pathways of B lymphocyte tolerance. *Immunol. Rev.* **43:**241.

13. Kiefer, H. 1975. Separation of antigen-specific lymphocytes. A new general method of releasing cells bound to nylon mesh. *Eur. J. Immunol.* **5:**624.

14. Venkataraman, M., and D. W. Scott. 1980. Cellular events in tolerance VIII. Analysis of the proliferative capacity of antigen-binding cells isolated from normal or tolerant spleens. *J. Immunol.* **124:**607.

15. Haas, W. 1975. Separation of antigen-specific lymphocytes. II. Enrichment of hapten specific antibody-forming cell precursors. *J. Exp. Med.* **141:**1015.

16. Veaux, D. L., B. L. Pike, and G. J. V. Nossal. 1981. Antibody production by single, hapten-specific B lymphocytes: an antigen-driven cloning system free of filler or accessory cells. *Proc. Natl. Acad. Sci. U. S. A.* **78:**7702.

17. Cambier, J. C., and M. J. Neale. 1982. Isolated phosphorylcholine binding lymphocytes. 1. Use of a cleavable crosslinking reagent for solid phase adsorbent isolation of functional antigen binding cells. *J. Immunol. Methods.* **51:**209.

18. Kettman, J. R., and Dutton, R. W. 1970. An *in vitro* primary immune response to 2,4,6-trinitrophenyl substituted erythrocytes: response against carrier and hapten. *J. Immunol.* **104:**1558.

19. Little, J. R., and Eisen, H. N. 1967. Conjugated and synthetic antigens. *Methods Immunol. Immunochem.* **1:**131.

20. Stanworth, D. R., and M. W. Turner. 1978. Immunochemical analysis of immunoglobulins and their subunits. *In* Handbook of Experimental Immunology. E. M. Weir, editor. Blackwell Scientific Publications, Oxford. 61.

21. Andres, C. M., A. Maddatena, S. Hudak, N. M. Young, and J. L. Claflin. 1981. Anti-phosphocholine hybridoma antibodies. II. Functional analysis of binding sites within three antibody families. *J. Exp. Med.* **154:**1584.

22. Kessler, S. W. 1975. Rapid isolation of antigens from cells with staphylococcal protein A-antibody adsorbent: parameters of interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115:**1617.

23. Baur, S., E. S. Vitetta, C. J. Sherr, I. Schenkein, and J. W. Uhr. 1971. Isolation of heavy and light chains of immunoglobulin from the surfaces of lymphoid cells. *J. Immunol.* **106:**1133.

24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680.

25. Dennert, G., R. Hyman, J. Lesley, and I. Trowbridge. 1980. Effects of cytotoxic hybridoma antibody specific for T200 glycoprotein on function lymphoid cell populations. *Cell. Immunol.* **53:**35.

26. Mishell, B. B., and S. M. Shiigi. 1980. Preparation of mouse cell suspensions. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, editors. W. H. Freeman & Co., San Francisco. 23.

27. Wetzel, G. D., and J. R. Kettman. 1981. Activation of murine B lymphocytes. III. Stimulation of B lymphocyte clonal growth with lipopolysaccharide and dextran sulfate. *J. Immunol.* **126:**723.

28. Young, C. W., and S. Hoda. 1964. Hydroxyurea: Inhibitory effects on DNA metabolism. *Science (Wash. D. C.).* **146:**1172.

29. Blyden, G., and R. E. Handschumacher. 1977. Purification and properties of human lymphocyte activating factor (LAF). *J. Immunol* **118:**1631.

30. Farrar, J. J., J. Fuller-Farrar, P. L. Simon, M. L. Hilfiker, B. M. Stadler, and W. Farrar. 1980. Thymoma production of T cell growth factor. *J. Immunol.* **125:**2555.

31. Fuller-Farrar, J., M. Hilfiker, W. Farrar, and J. Farrar. 1981. PMA enhances the production of interleukin 2. *Cell. Immunol.* **58:**156.

32. Elkins, K. L., and J. C. Cambier. 1983. Constitutive production of a factor supporting B lymphocyte differentiation by a T cell hybridoma. *J. Immunol.* In press.

33. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126:**423.

34. Hunig, T. H., A. Schimpl, and E. Wecker. 1977. Mechanism of T-cell help in the immune response to soluble protein antigens. I. Evidence for *in situ* generation and action of T-cell replacing factor during the anamnestic response to dinitrophenyl keyhole limpet hemocyanin *in vitro. J. Exp. Med.* **145:**1216.

35. Harwell, L., J. W. Kappler, and P. Marrack. 1976. Antigen-specific and nonspecific mediators of T cell/B cell cooperation. III. Characterization of the nonspecific mediator(s) from different sources. *J. Immunol.* **116:**1397.

36. Cambier, J. C., F. S. Ligler, J. W. Uhr, and E. S. Vitetta. 1978. Blocking of primary in vitro antibody responses to thymus-independent anf thymus dependent antigens with antiserum specific for IgM or IgD. *Proc. Natl. Acad. Sci. U. S. A.* **75:**432.

37. Haskill, J. S. 1969. Density distribution analysis of antigen-sensitive cells in the rat. *J. Exp. Med.* **130:**877.

38. Gorzynski, R. M., and M. Feldman. 1975. B cell heterogeneity—difference in the size of B lymphocytes responding to T dependent and T independent antigens. *Cell. Immunol.* **19:**88.

39. Cambier, J. C., J. R. Kettman, E. S. Vitetta, and J. W. Uhr. 1977. B cell tolerance. II. Trinitrophenyl human gamma globulin-induced tolerance in adult and neonatal murine

B cell responsive to thymus dependent and independent forms of the same hapten. *J. Exp. Med.* **145**:778.

40. Tite, J. R., S. Marshall-Clarke, and J. H. L. Playfair. 1978. Differential tolerance of thymus-independent and thymus dependent antibody responses. *Immunology.* **34**:337.

41. Fidler, J. M. 1979. The induction of hapten-specific immunological tolerance and immunity in B lymphocytes. VI. Differential tolerance susceptibility in adult spleen as a function of B cell maturational level. *J. Exp. Med.* **150**:491.

42. Boswell, H. S., A. Ahmed, I. Scher, and A. Singer. 1980. Role of accessory cells in B cell activation. II. The interaction of B cells with accessory cells results in the exclusive activation of an Lyb 5⁺ B cell subpopulation. *J. Immunol.* **125**:1340.

43. Quintans, H., and H. Cosenza. 1976. Antibody response to phosphorylcholine *in vitro.* II. Analysis of T-dependent and T-independent responses. *Eur. J. Immunol.* **5**:399.

44. Jennings, J. J., and M. B. Rittenberg. 1976. Evidence for separate subpopulations of B cells responding to T-independent and T-dependent immunogen. *J. Immunol.* **177**:1749.

45. Ahmed, A., and I. Scher. 1979. Murine B cell heterogeneity defined by anti-Lyb 5, an alloantiserum specific for a late-appearing B lymphocyte subpopulation. *In* B Lymphocytes in the Immune Response. M. Cooper, D. E. Mosier, E. S. Vitetta, and I. Scher, editors. Elsevier/North-Holland, New York. 117-124.

46. Zitron, I. M., D. E. Mosier, and W. E. Paul. 1977. The role of surface IgD in the response to thymic-independent antigens. *J. Exp. Med.* **146**:1707.

47. Mosier, D. E., I. Scher, and W. E. Paul. 1976. In vitro responses of CBA/N mice. Spleen cells of mice with an X-linked defect that precludes immune responses to several thymus dependent antigens can respond to TNP-lipopolysaccharide. *J. Immunol.* **117**:1363.

48. Andersson, J., A. Coutinho, and F. Melchers. 1977. Frequencies of mitogen-reactive B-cells in the mouse. I. Distribution in different lymphoid organs from different inbred strains of mice at different ages. *J. Exp. Med.* **145**:1511.

49. Scott, D. W., and C. Alexander. 1980. B-cell subsets responsive to fluorescein-conjugated antigens. II. "Cross priming" and its elimination by bromodeoxyuridine and light. *Cell. Immunol.* **53**:376.

50. Mosier, D. E. 1978. Induction of B cell priming by neonatal injection of mice with thymic-independent (type 2) antigens. *J. Immunol.* **121**:1543.

51. Andersson, J., and F. Melchers. 1981. T cell-dependent excitation and stimulation of B cells. *In* B Lymphocytes in the Immune Response: Functional, Developmental, and Interactive Properties. N. Klinman, D. Mosier, I. Scher, and E. Vitetta, editors. Elsevier/North-Holland, New York. 307-314.

52. Sieckman, D. G., I. Scher, R. Asofsky, D. E. Mosier, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. II. A thymus independent response by a mature subset of B lymphocytes. *J. Exp. Med.* **148**:1628.