

ACTIVATION OF MURINE B LYMPHOCYTES BY  
ANTI-IMMUNOGLOBULIN IS AN INDUCTIVE SIGNAL  
LEADING TO IMMUNOGLOBULIN SECRETION\*

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B cells in culture respond to anti-immunoglobulin reagents (anti-Ig) by blast transformation and DNA synthesis (1, 2). Anti-Ig activates by binding to and cross-linking cell surface Ig (3-5). Because surface Ig has been shown to function as the receptor for antigen, accounting for the specificity of the antibody response by the process of clonal selection, it is not unreasonable to assume that anti-Ig acts as an antireceptor antibody to mimic polyclonally the effects of antigen on antigen-sensitive cells. However, the relevance of polyclonal B cell activation by anti-Ig to the antibody response depends on showing that anti-Ig can be an inductive signal leading to high-rate Ig synthesis and secretion. On first appraisal, the evidence to the contrary is striking: anti-Ig is unique among polyclonal B cell activators owing to the complete absence of accompanying polyclonal antibody secretion (2, 6, 7). But many antigens, including oligovalent protein antigens whose effects at the B cell surface would most closely resemble those of anti-Ig, are also incapable of inducing antibody synthesis in the absence of primed, antigen-specific, helper T cells. According to one model for the role of helper T cells in the antibody response, T cells upon antigen recognition secrete an antigen-nonspecific helper factor that together with antigen enables B cells to differentiate into antibody-secreting cells (8). In support of this model, Kishimoto et al. (9) showed that rabbit mesenteric lymph node cells could be induced to secrete IgG by treatment with goat anti-Fc (IgG) followed by supernatant culture fluid from primed cells incubated with antigen.

With this model of T-B cell collaboration in mind, we added a rich source of antigen-nonspecific factor(s) (the supernatant fluid of a 24-h culture of spleen cells activated with concanavalin A [Con A]<sup>1</sup> [SN]) to cultures of murine B lymphocytes activated by anti-Ig antibodies which were attached covalently to polyacrylamide beads (anti-Ig beads). We found that the B cells differentiated to produce large numbers of cells secreting Ig at a high rate (10). In this report, we show that anti-Ig antibodies in soluble form are as active as anti-Ig beads in inducing polyclonal Ig secretion in the presence of SN, if one has removed the Fc portion from the anti-Ig.

This polyclonal model for the antibody response has enabled us to begin looking

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<sup>1</sup> *Abbreviations used in this paper:* anti-Ig beads, polyacrylamide beads covalently coupled to anti-Fab antibodies; C, complement; Con A, concanavalin A; FCS, fetal calf serum; L, Ig light chain; LPS, lipopolysaccharide; PFC, plaque-forming cell; PHA, phytohemagglutinin; SN, supernatant culture fluid from spleen cells incubated with Con A for 24 h; V<sub>H</sub>, variable region of Ig heavy chain.

directly at the roles of the two major surface Ig isotypes, IgM and IgD, in B cell activation. Because class-specific  $F(ab')_2$  anti- $\mu$  antibodies are at least as effective as  $F(ab')_2$  anti-Fab antibodies, it would appear that surface IgD does not need to be involved in one pathway of B cell activation leading from a resting B cell to a high-rate Ig-secreting cell.

### Materials and Methods

*Animals.* DBA/2J mice of both sexes were obtained from The Jackson Laboratory, Bar Harbor, Maine, and were used between 8 and 16 wk of age.

*Antibodies.* The anti-IgM antibodies were raised against MOPC 104E ( $\mu$ ,  $\lambda$ ) protein (Biogenetics Laboratory Products, Litton Bionetics Inc., Kensington, Md.) and isolated on TEPC 183 ( $\mu$ ,  $\kappa$ ) Sepharose. TEPC 183 protein was partially purified from ascites fluid by precipitation with boric acid (11), absorption with protein A-Sepharose (Pharmacia Fine Chemicals Div. Pharmacia, Inc., Piscataway, N. J.), and gel filtration in Sephadex G-200. The antibodies eluted from TEPC 183 Sepharose were absorbed on Sepharose conjugated to the boric acid supernatant solution of TEPC 183 ascites. These anti- $\mu$  antibodies gave a single band near the origin in immunoelectrophoresis gels developed with anti-whole mouse serum. Double diffusion gels showed a band of identity between TEPC 183 protein and mouse serum and no reaction with MOPC 315 ( $\alpha$ ,  $\lambda$ ), UPC 10 ( $\gamma 2a$ ,  $\kappa$ ), or normal mouse IgG prepared by ion-exchange chromatography. The anti-Fab antibodies were prepared as described (10), and gave bands of identity in double diffusion gels between whole mouse serum, UPC 10, mouse IgG, and  $F(ab')_2$  mouse IgG. The last reagent was prepared by the same scheme used to make normal rabbit IgG  $F(ab')_2$ , viz., isolation of IgG from serum on protein A-Sepharose at pH 8, elution with 0.5 M sodium acetate at pH 3.0, digestion at 37°C at pH 4.3–4.4 in 0.5 M acetate for 18 h with pepsin (Sigma Chemical Co., St. Louis, Mo.) at 1:50 wt/wt pepsin to Ig, and absorption with protein A-Sepharose followed by gel filtration in Sephadex G-200.  $F(ab')_2$  fragments were prepared from purified antibodies by the same scheme.  $F(ab')_2$  anti-Fab was reduced and alkylated as described (3), and the Fab' fragments were purified by gel filtration in Sephadex G-100.

Anti-Ig beads were prepared by covalently coupling  $F(ab')_2$  anti-Fab antibodies to 100- $\mu$ m polyacrylamide beads as described (10).

*Cell Fractionation and Culture.* B lymphocytes were isolated from spleen cell suspensions as rosettes with anti-Ig-coated sheep red cells (see below, reverse plaque assay) through high density ( $\rho = 1.092$ ) Ficoll/Hypaque (10). These B cell-enriched populations were always over 90% surface Ig positive by immunofluorescence and responded very poorly or not at all to phytohemagglutinin (PHA) and Con A in a proliferative assay. However, they contained 3–7% phagocytic cells as estimated by latex-bead ingestion (12).

In some experiments, T cells were killed by incubation with monoclonal (F7D5) IgM anti-Thy 1.2 (13; a gift from Dr. Phil Lake, University College London, London) followed by agarose-absorbed guinea pig complement.

Isolated B cells were cultured in flat-bottomed cluster dish wells (Costar 3596; Costar Data Packaging, Cambridge, Mass.) at  $10^5$  cells/200  $\mu$ l RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM glutamine, and 20% fetal calf serum (FCS) (Grand Island Biological Co.) in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C.

*Con A SN.* SN was prepared by incubating whole spleen cells for 24 h in Petri dishes at  $10^7$  cells/ml in culture medium with 20% FCS and 4  $\mu$ g/ml Con A (Miles-Yeda, Rehovot, Israel). Excess Con A was removed from the supernatant culture fluid by passage through Sepharose coupled to purified rabbit anti-Con A antibodies. The SN was filtered and stored at –70°C. 100  $\mu$ l of SN was added to 100  $\mu$ l of cell suspension with other additions to yield a final SN concentration of approximately 50%. Lower concentrations of SN gave smaller responses.

*Cell Counts.* Recovery of cultured cells was estimated with a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.) using an erythrocyte-lysing agent designed for leukocyte blood counts (B3145-6A; Scientific Products Inc., State College, Pa.). Total cell counts with this

procedure included a variable percentage of recently dead cells that had not yet disintegrated, as was determined separately by trypan blue dye exclusion.

*Reverse Hemolytic Plaque Assay for Ig-secreting Cells.* As described (10), the assay uses red cells coated with a chemically prepared rabbit hybrid antibody containing one active site against sheep red cells and the other against mouse Fab. In lipopolysaccharide (LPS)-activated microcultures on days 3 and 4, the assay detects as direct plaques by a liquid monolayer technique 50–100% of the cells that show bright intracellular fluorescence with fluorescein-labeled anti-Fab in fixed smears. Both an IgM (TEPC 183) and an IgG<sub>2a</sub> (UPC 10) plasmacytoma produce direct plaques, but the efficiency for detection of secreting plasma cells of other isotypes is not known. Triplicate cultures were pooled, washed three times, and assayed in duplicate at a dilution yielding 50–200 PFC per slide.

*Other Reagents.* LPS (Westphal) from *Escherichia coli* 055:B5 (Difco Laboratories, Detroit, Mich.) was used at 50 µg/ml. PHA (HA16; Burroughs-Wellcome Research Triangle Park, N.C.) was used at 2 µg/ml. Ficoll was from Pharmacia Fine Chemicals, and Hypaque was from Winthrop Laboratories, New York.

*Electron Microscopy.* Cells from each treatment group were pooled and washed with phosphate-buffered saline. Dead cells and debris were removed by centrifugation over high density ( $\rho = 1.092$ ) Ficoll/Hypaque. After washing, the lymphocytes were fixed for 2 h at room temperature in 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3. The samples were postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated with a graded series of ethanol solutions, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and studied and photographed with a JEOL 100S electron microscope (JEOL USA, Electron Optics Div., Medford, Mass.) at 60 kV.

## Results

*Differentiation to PFC of Anti-Ig-activated B Cell Cultures Can Be Induced by Addition of SN.* Isolated mouse B lymphocytes can be activated by rabbit F(ab')<sub>2</sub> anti-mouse Fab antibodies, as shown by stimulation of DNA synthesis measured by radioisotope incorporation (data not shown). The addition of SN to these cultures results in the differentiation of large numbers of activated B cells into Ig-secreting cells as measured by a reverse plaque assay (Fig. 1). This polyclonal plaque-forming cell (PFC) response to F(ab')<sub>2</sub> anti-Fab plus SN is comparable in size with the PFC response to LPS, although it peaks a day later. It is strictly dependent on SN because F(ab')<sub>2</sub> anti-Fab by itself merely suppresses background plaque production (fewer than 10 PFC/culture). SN by itself or with control F(ab')<sub>2</sub> normal rabbit Ig induces a small PFC response which can be reduced but not eliminated by treating spleen cells with anti-Thy 1.2 plus complement (C) before isolation of B cells as rosettes with anti-Ig-coated erythrocytes (see below).

*Addition of SN Can Be Delayed.* SN can be added to the B cell cultures 24 h after addition of F(ab')<sub>2</sub> anti-Fab without changing the kinetics of the PFC response (Fig. 2). Delaying addition of SN to 48 h results in a 24-h delay in the response. Delaying addition of F(ab')<sub>2</sub> anti-Fab by 24 h delays the response by 24 h (data not shown). We conclude that anti-Ig and SN act sequentially on the cultures, and that anti-Ig by itself initiates a sequence of events which requires the presence of SN 24 h later in order to result in the maturation of B cells into Ig-secreting cells on days 4 and 5.

*Electron Microscopy.* The delay of at least 40 h between addition of SN to anti-Ig-activated B cells and the appearance of PFC indicates that SN is required for an early event in the differentiation of a B lymphoblast to an Ig-secreting cell, rather than the final secretory step. In order to see whether the presence of SN resulted in morphological differentiation, we compared the ultrastructure of cells recovered after 4 d in culture with F(ab')<sub>2</sub> anti-Fab in the presence or absence of SN (Fig. 3). As expected,

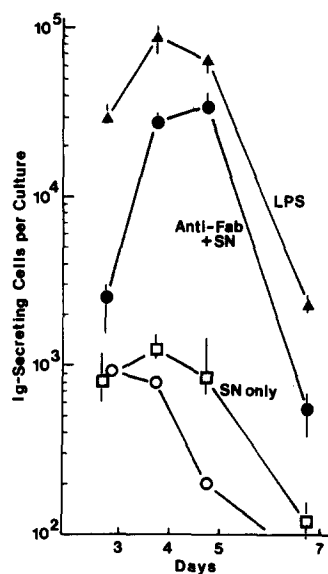


FIG. 1. Time-course of the polyclonal PFC response of  $10^5$  B lymphocytes to  $F(ab')_2$  anti-Fab ( $50 \mu\text{g}/\text{ml}$ ) plus SN ( $\bullet$ ), control  $F(ab')_2$  normal rabbit IgG plus SN ( $\circ$ ), SN only ( $\square$ ), and LPS ( $\blacktriangle$ ). Cultures with no additions or with only  $F(ab')_2$  anti-Fab had fewer than  $10^2$  PFC/culture. The error bars show the range around the median of three independent points from the same experiment. Each point is the mean of two PFC determinations of a pool of three cultures.

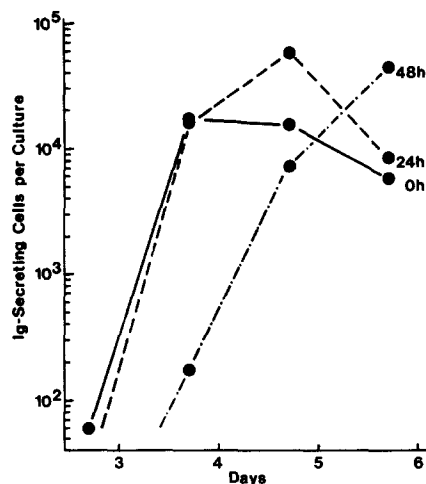


FIG. 2. The effect of delayed addition of SN on the polyclonal PFC response to  $F(ab')_2$  anti-Fab ( $40 \mu\text{g}/\text{ml}$ ). SN was added at the beginning of culture (—), or half the culture fluid was removed and replaced by SN at 24 h (---) or at 48 h (-.-). Cultures with SN only had maximal PFC on day 3.7: 540 PFC (0 h), 630 PFC (24 h), 80 PFC (48 h).

the majority of cells in both groups are blast cells with large, euchromatic nuclei, prominent nucleoli, and many polyribosomes. The two groups differ in several morphological characteristics. Most but not all cells cultured with SN contain considerable rough endoplasmic reticulum, the cellular machinery for high-rate synthesis of a protein destined for secretion; some plasmablasts demonstrate prominent

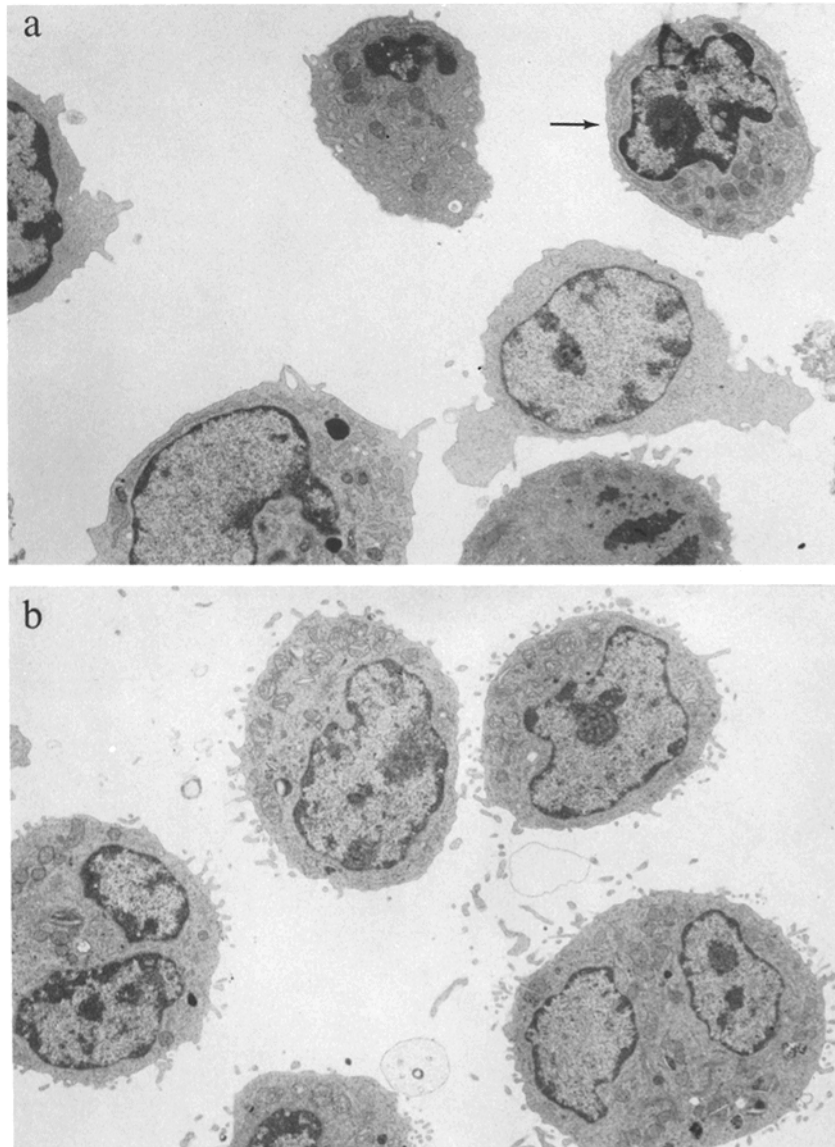


FIG. 3. Electron micrographs of B lymphocytes recovered after 4 d of culture with F(ab')<sub>2</sub> anti-Fab (20 µg/ml) with SN (a) or without SN (b). The arrow points to a cell with extensive rough endoplasmic reticulum. × 5,250.

Golgi apparatuses, eccentric nuclei, and extensive rough endoplasmic reticulum. Cells cultured with F(ab')<sub>2</sub> anti-Fab but without SN contain little or no rough endoplasmic reticulum and include no plasmacytoid cells (Fig. 3 b).

*The PFC Response to Anti-Ig Plus SN Appears to Be T Cell Independent.* Although the production of an active SN requires Con A and the presence of T lymphocytes (data not shown), the SN, once produced, does not appear to require T cells to mediate its effects on anti-Ig-activated B cells. Table I shows the lack of effect of T cell depletion

TABLE I  
*T Cell Independence of the Response to Anti-Ig Plus SN*

Addition to culture	Whole spleen	Anti-Thy 1.2 plus C	B cell rosettes	Anti-Thy 1.2 plus C, then B cell rosettes
	<i>PFC/culture*</i>			
None	70 (9)	100 (3)	80 (4)	50 (3)
F(ab') <sub>2</sub> anti-Fab (20 µg/ml)	10 (22)	10 (28)	10 (18)	10 (23)
SN	3,460 (139)	2,500 (47)	2,250 (43)	500 (12)
F(ab') <sub>2</sub> anti-Fab plus SN	15,420 (218)	53,330 (145)	41,670 (115)	61,250 (129)
LPS	50,010 (108)	44,340 (107)	79,580 (264)	77,500 (282)
PHA	180 (36)	110 (7)	130 (10)	10 (3)
Con A	300 (121)	680 (31)	420 (40)	50 (2)

\* The total number of cells recovered per culture  $\times 10^{-3}$  after 4 d is shown in parenthesis. Each culture initially contained  $100 \times 10^3$  viable cells.

on the response to anti-Ig plus SN. Treatment of spleen cells with monoclonal (F7D5) anti-Thy 1.2 plus C or isolation of B cells as rosettes with anti-Ig-coated sheep red cells abolishes the proliferative response to PHA and depresses the response to Con A by more than 90% as measured by an isotope incorporation assay at 48 h (10, 13). Either treatment enhances the PFC response to anti-Ig plus SN while depressing the proliferative responses to Con A, PHA, and SN alone as measured by cell recovery after 4 d (Table I, numbers of recovered cells are shown in parentheses). The two treatments applied sequentially abolish the lectin responses completely without affecting the anti-Ig plus SN response. There is still a residual PFC response to SN by itself in the cells receiving both treatments which could be a result entirely of a small proportion of B cells which were activated in the mouse before our experiments. Table I also shows that SN greatly enhances B cell proliferation or survival of proliferating cells in cultures containing anti-Ig ( $129 \times 10^3$  vs.  $23 \times 10^3$  cells recovered per culture).

*The Response to Anti-Ig Plus SN Requires Cross-Linkage of Surface Ig.* Other workers have shown that the proliferative response of rabbit and mouse B lymphocytes to anti-Ig reagents requires cross-linkage of cell surface Ig molecules because divalent F(ab')<sub>2</sub> reagents are effective whereas monovalent Fab or Fab' reagents are not (3-5). In order to determine whether cross-linkage is required for the polyclonal PFC response to anti-Ig plus SN, a monovalent Fab' anti-mouse Fab reagent was prepared by limited reduction, alkylation, and gel filtration of our F(ab')<sub>2</sub> reagent. The monovalent reagent fails to induce PFC or cell proliferation with SN present at any concentration from 0.01 to 200 µg/ml. It retains its ability to bind to surface Ig, however, because it inhibits the response to F(ab')<sub>2</sub> anti-Fab plus SN when added in excess; monovalent Fab' anti-Fab at 200 µg/ml nearly completely inhibits the response to 50 µg/ml of divalent F(ab')<sub>2</sub> anti-Fab plus SN (Fig. 4). In a separate experiment, the same concentration of monovalent Fab' anti-Fab caused only a 35% reduction in the polyclonal PFC response to LPS (59,200-38,700 PFC/culture). Therefore, it is likely that the monovalent reagent inhibits the PFC response by preventing cross-linking by competing with the divalent reagent for antigenic determinants on surface Ig, in the same way that it would inhibit precipitation of Ig by anti-Ig in solution.

*The Response to F(ab')<sub>2</sub> Anti-Fab Is Sensitive to Fc-mediated Inhibition.* Intact, undigested

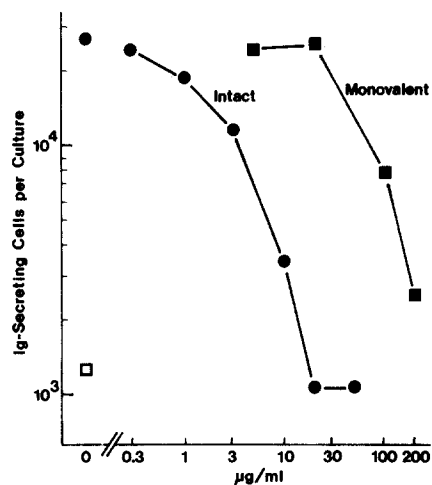


FIG. 4. Inhibition of the polyclonal response to  $F(ab')_2$  anti-Fab ( $50 \mu\text{g/ml}$ ) plus SN by monovalent Fab' anti-Fab (—■—) or by intact, undigested anti-Fab (—●—).

TABLE II  
*The Inhibitory Effect of Intact Anti-Fab on the Response to  $F(ab')_2$  Anti-Fab Plus SN*

Concentration of intact anti-Fab $\mu\text{g/ml}$	Response to SN	Response to $F(ab')_2$ anti-Fab ( $50 \mu\text{g/ml}$ ) plus SN
None	560 (38)	20,000 (116)
1	422 (36)	3,830 (95)
5	110 (28)	830 (73)
25	20 (31)	<30 (34)
100	<10 (47)	<30 (35)

\* The total number of cells recovered per culture  $\times 10^{-3}$  is shown in parentheses.  $10^4$  isolated B cells were cultured for 4 d.

anti-Fab antibodies fail to produce a polyclonal PFC response in the presence of SN at any concentration tested (Table II). Intact antibodies also inhibit completely the PFC response to the  $F(ab')_2$  fragment (Table II and Fig. 4). Unlike the effect of monovalent anti-Ig, this effect is not likely to be the result of competition between  $F(ab')_2$  and intact antibody for surface Ig molecules: the concentration of  $F(ab')_2$  used in Table II and Fig. 4 ( $50 \mu\text{g/ml}$ ) is two- to fivefold optimal for the PFC response (compare Fig. 6), yet 1–3  $\mu\text{g/ml}$  of intact antibody inhibits the response by >50% and 20  $\mu\text{g/ml}$  of intact antibody inhibits the response completely.

*LPS Cannot Substitute for Either Anti-Fab or SN in the Polyclonal PFC Response.* To address the possible contribution of LPS contamination to the responses reported here, LPS was added at concentrations from 5 ng/ml to 50  $\mu\text{g/ml}$  to cultures containing SN,  $F(ab')_2$  anti-Fab, or both agents. The result (Fig. 5) clearly distinguishes the effects of SN or  $F(ab')_2$  anti-Fab from those of LPS. The response to LPS plus SN is simply additive at best, with no suggestion of synergy, whereas  $F(ab')_2$  anti-Fab strongly inhibits the polyclonal PFC response to LPS, as has been reported for other anti-Ig reagents (14, 15). Also, by the Limulus assay neither the antibodies

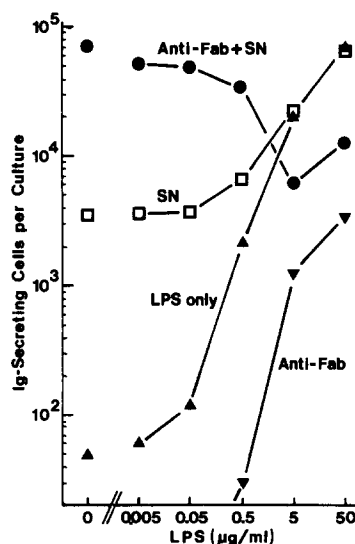


FIG. 5. LPS at any dose cannot substitute for anti-Fab or SN in the response to anti-Fab plus SN. The figure shows the polyclonal PFC response of  $10^5$  B cells on day 4 to various doses of LPS alone ( $\blacktriangle$ ) or together with F(ab')<sub>2</sub> anti-Fab (20 µg/ml) ( $\blacktriangledown$ ), SN ( $\square$ ), or F(ab')<sub>2</sub> anti-Fab (20 µg/ml) plus SN ( $\bullet$ ).

nor the SN contained more endotoxin than the culture medium with 20% FCS (5–25 ng/ml) (10). High concentrations of LPS interfere by an unknown mechanism with the response to anti-Fab plus SN.

*Class-specific Anti- $\mu$  Antibodies Are as Effective as Anti-Fab Antibodies in Inducing Polyclonal Ig Secretion.* Because of shared variable region of the Ig heavy chain ( $V_H$ ) and Ig light chain (L) antigenic determinants, anti-Fab antibodies against mouse IgG Fab would be expected to react equally well with each of the two major classes of cell surface Ig, IgM, and IgD. We have found that class-specific F(ab')<sub>2</sub> anti- $\mu$  antibodies are at least as effective as anti-Fab antibodies in inducing B cell proliferation and differentiation to Ig secretion in the presence of SN (Fig. 6). As with anti-Fab antibodies, the Fc portion of anti- $\mu$  antibodies must be removed. Interestingly, both F(ab')<sub>2</sub> anti- $\mu$  and F(ab')<sub>2</sub> anti-Fab at suboptimal concentrations inhibited the small response to SN alone.

The anti- $\mu$  antibodies were elicited in rabbits by MOPC 104E protein ( $\mu$ ,  $\lambda$ ), then isolated on TEPC 183 protein ( $\mu$ ,  $\kappa$ ), and finally absorbed with IgM-depleted TEPC-183 ascites fluid. Consequently they should not cross-react with surface IgD on the basis of  $V_H$  or L chain determinants. In addition, the purified anti- $\mu$  antibodies used in these experiments were specific for surface IgM and failed to react with surface IgD by immunoprecipitation and SDS gel electrophoresis of <sup>125</sup>I-radiolabeled membrane proteins (R. Pollock and M. Mescher. Personal communication.). Therefore, direct involvement of surface IgD with ligand is not required for this B cell activation pathway.

### Discussion

The experiments described here are consistent with the existence of a B cell activation pathway which occurs in two discrete stages, in response to distinct and



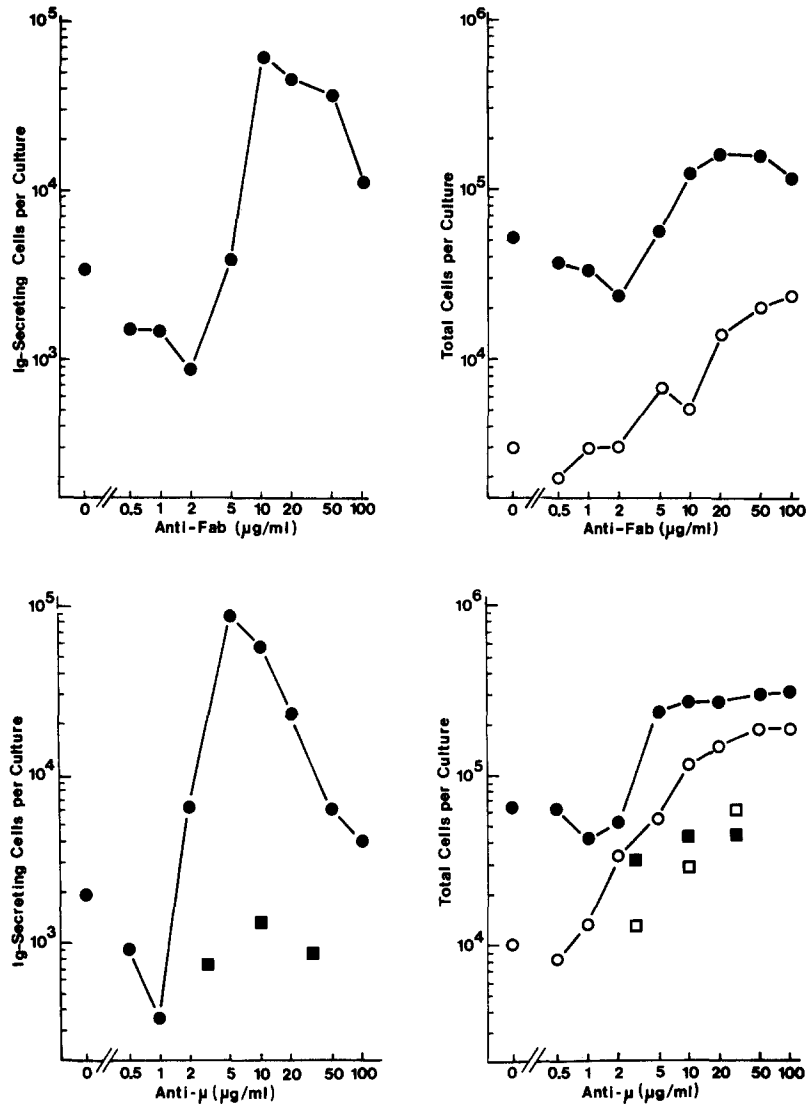


FIG. 6. The polyclonal PFC and proliferative responses to  $F(ab')_2$  anti-Fab and  $F(ab')_2$  anti- $\mu$  plus SN (●) or without SN (○) as a function of anti-Ig dose. The response to intact anti- $\mu$  plus SN (■) or without SN (□) is also shown.  $10^5$  B lymphocytes were cultured for 4 d. Cultures without SN had fewer than  $10^2$  PFC.

sequential signals. The first signal is delivered by redistribution of surface Ig and results over 24–48 h in blast transformation, DNA synthesis, limited proliferation, and the ability to respond to a second signal in SN. The second signal which is present in SN results in more extensive proliferation, or survival of proliferating cells, and differentiation to high-rate Ig synthesis and secretion. No differentiation to Ig secretion occurs in the absence of SN, and SN appears to act only on anti-Ig-activated, not resting, B cells. We have not yet excluded more complicated interpretations involving collaboration between B cell subsets, one of which responds to anti-Ig enabling

another to proliferate and to differentiate to PFC in response to SN, but for purposes of discussion we shall assume that the cells which respond to anti-Ig include the precursors of the PFC which appear later when cultured in SN.

We consider the PFC response to anti-Ig plus SN to be a polyclonal model for a thymus-dependent antibody response. Anti-Ig substitutes polyclonally for antigen in inducing blast transformation by redistribution of surface Ig. SN is a rich source of antigen-nonspecific helper factor(s) which can replace antigen-specific T helper cells in *in vitro* antibody responses to heterologous erythrocytes or hapten-protein conjugates (16). The important finding by Schimpl and Wecker that T cell-replacing supernatant media of activated T cells could be added to nude spleen cell cultures 48 h after antigen without changing the kinetics of the appearance of PFC led them to propose that the antibody response involves distinct, sequential signals to B cells (17). Subsequent experiments by Hünig et al. (18) and by Dutton (19) established that B cells respond to antigen with DNA synthesis before T cell help is added. In this Dutton-Schimpl scheme for T-B collaboration, the nonspecific helper factor is produced locally when T cells recognize carrier determinants, and acts only at short range *in vivo* to preserve the specificity of T-B collaboration demonstrated by the carrier effect. We should point out that the proliferative and PFC responses to anti-Ig appear to be limited to a subset of B cells that is missing in CBA/N mice, which have an X-linked defect in B cell development, and in very young mice of normal strains (5, 10, 20). Because B cells from these two sources respond quite well to thymus-dependent antigens, there are likely to be other pathways of thymus-dependent B cell activation than the one under study here.

We have not found any striking differences in the PFC response in the presence of SN between B cell cultures activated by anti-Ig on polyacrylamide beads (10) and B cells cultures activated by soluble  $F(ab')_2$  anti-Ig. As we reported (10), the proliferative response to anti-Ig beads is more intense and less fastidious than the response to soluble anti-Ig with regard to requirements for serum, 2-mercaptoethanol, or adherent cells, and Fc-mediated inhibition. However, cultures activated by either means differentiate to produce large numbers of Ig-secreting cells in the presence of unfractionated SN under the conditions reported here, *viz.*, low cell density with 2-mercaptoethanol. Note that isolation of B cells as anti-Ig rosettes, a procedure that briefly exposes B cells to anti-Ig in a matrix on the red cell surface, is not a necessary step in the response to soluble anti-Ig plus SN (Table I).

Finding the PFC response to soluble anti-Ig has enabled us to show that cross-linking of surface Ig is required for activation leading to Ig secretion, as well as for the proliferative response (4-6), because the monovalent Fab' fragments failed to activate and blocked activation by the  $F(ab')_2$  fragments. Although cross-linkage leads to capping and endocytosis of surface Ig, the PFC response to anti-Ig beads plus SN shows that activation is probably a surface event, not dependent upon internalization of antigen-receptor complexes.

The Fc portion of rabbit anti-Ig strongly inhibits mouse B cell activation by anti-Ig (Table II and Fig. 4) and accounts for our earlier failure (6) and perhaps the failure of others (21) to activate mouse B cells with anti-Ig in soluble form. Scribner et al. (22) studied the age dependence of Fc-mediated inhibition of the proliferative response to rabbit anti-IgM, and concluded that the inhibition was Fc receptor mediated and intrinsic to the B cell itself. On the other hand, mouse cells seem to be indifferent to

the Fc of goat anti- $\mu$  (23); goat Fc also binds less avidly than rabbit Fc to Fc receptors on human blood leukocytes (24). This Fc-dependent inhibition of B cell activation may be a regulatory mechanism for shutting down recruitment of additional B cell clones in the presence of excess IgG antibody (25).

The recent finding that the majority of human and mouse lymphocytes have surface IgD as well as IgM led to speculation that the two classes might have distinct regulatory roles, so that the susceptibility of a particular B cell to certain antigen-associated triggering or tolerance signals might be determined by the relative amounts of sIgM and sIgD on the cell membrane (26). Experiments designed to test such hypotheses have been reviewed (27-29). Sidman and Unanue (30) and Sieckmann et al. (31) have shown by cell sorting experiments that the subset of B cells which responds to anti- $\mu$  antibodies by DNA synthesis has relatively large amounts of sIgD. Nevertheless, our experiments show that surface IgD involvement is not necessary as an inductive signal over the entire pathway leading from a resting B cell through proliferation and differentiation to an Ig-secreting plasmablast because class-specific anti- $\mu$  antibodies are as effective as anti-Fab antibodies in this process. Further experiments in progress with specific anti- $\delta$  antibodies may determine whether surface IgD can have an inductive or modulating role at any stage in the pathway.

### Summary

Cultures of isolated mouse splenic B lymphocytes activated by the divalent  $F(ab')_2$  fragment of purified rabbit anti-mouse Fab or class-specific anti-mouse IgM antibodies can be driven on to high-rate Ig secretion by the addition of the supernatant fluid of a 24-h culture of concanavalin A-activated spleen cells (SN). The polyclonal antibody response to anti-Ig plus SN is comparable in magnitude with the lipopolysaccharide response as measured in a reverse plaque assay. The addition of SN can be delayed for 24 h after addition of anti-Ig without changing the kinetics of the response. Addition at 48 h delays the response by 24 h. The response to  $F(ab')_2$  anti-Fab plus SN is sensitive to Fc-dependent inhibition because intact anti-Fab antibodies inhibit strongly at relatively low concentrations. The monovalent Fab' fragment fails to induce Ig secretion, indicating that cross-linkage of surface immunoglobulin is required. Although the production of active SN is T cell dependent, the response to anti-Ig plus SN is T independent. These findings are interpreted as a polyclonal model of a thymus-dependent antibody response.

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