

ACTIVATION OF MOUSE LYMPHOCYTES BY ANTI- IMMUNOGLOBULIN

II. A Thymus-Independent Response by a Mature Subset of B Lymphocytes*

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It is now clear that the binding of antigen by specific receptors of thymus-independent (B) lymphocytes is an important step in the physiologic process by which such cells are activated (1, 2). However, the precise role of antigen-specific membrane receptors is still unknown. It has been proposed that the binding of antigens by membrane immunoglobulin directly generates some type of transmembrane signal which is critical to lymphocyte activation (3-7). Others (8-11) have suggested that the interaction of antigen with immunoglobulin receptors, in and of itself, has no effect on the cell but that the receptor-ligand interaction serves to concentrate an intrinsically stimulatory molecule on the cell surface. The latter theory, which has been referred to as the "one non-specific signal" theory (10, 11), predicts that anti-immunoglobulin antibodies, in the absence of "help" from thymus-dependent (T) lymphocytes or from polyclonal activators, should have no effect on B lymphocytes. Consequently, a detailed evaluation of the behavior of B lymphocytes after their exposure to anti-immunoglobulin (anti-Ig)¹ antibodies should provide important information to aid in the choice between these hypotheses.

It has previously been shown that anti-Ig antibodies will induce proliferation by lymphocytes from several species, including rabbits (12), humans (13-16), pigs (17),

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¹ Abbreviations used in this paper: FATS, anti-thymocyte serum; Con A, Concanavalin A; ACS, fluorescence activated cell sorter; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid; [³H]TdR, methyl[³H]thymidine; Ig, immunoglobulin; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; *nu/nu*, athymic nude mice; *nu/+*, heterozygous littermates; NWSM, *Nocardia* water-soluble mitogen; NWT, nylon wool T lymphocytes; PFC, plaque-forming cells; PHA, phytohemagglutinin-P; RE, reference endotoxin; SRBC, sheep erythrocytes.

and chickens (18, 19). Recently, conditions have been described under which mouse lymphocytes will proliferate in response to anti-Ig (20–22). Studies from our laboratory (22) indicate that lymphocytes from young adult mice will synthesize DNA when exposed to specifically purified anti- μ or anti- κ antibodies. Both deaggregated antibodies and F(ab')₂ fragments were equally effective, and serum is not required for these responses.

In this communication, we show that the response of mouse lymphocytes to anti- μ and anti- γ, κ is a function of B lymphocytes, that it does not require the presence of T lymphocytes, and that adherent cells are not required for the response. These results thus suggest that the activation of B cells by anti-Ig is a direct consequence of its interaction with membrane Ig and is independent of auxiliary signals from either T lymphocytes or macrophages.

Finally, our data also indicate that anti-Ig stimulates a mature subset of B lymphocytes. This is based on the finding that responsiveness to anti- μ or to anti- γ, κ does not appear until 4 wk of age and that lymphocytes from mice with the CBA/N X-linked immune defect in B-lymphocyte function (23) fail to respond to anti-Ig.

Materials and Methods

Animals. (C57BL/6 \times DBA/2J)F₁ (BDF₁/J) and CBA/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. (C57BL/6 \times DBA/2N)F₁ (BDF₁/N) and (CBA/N \times DBA/2N)F₁ mice were obtained from the Division of Research Services, National Institutes of Health. Athymic nude (*nu/nu*) mice and heterozygous littermates (*nu/+*), members of a 10th generation backcross to BALB/c, were obtained at 10 wk of age from Charles River Breeding Laboratories, Wilmington, Mass. All mice were used at 2–3 mo of age unless noted otherwise.

Anti-Immunoglobulin (Anti-Ig) Antibodies. Affinity column-purified goat anti-mouse Ig specific for μ -heavy chains (anti- μ) or for γ -heavy and κ -light chains (anti- γ, κ) were prepared and assayed for specificity as previously described (22).

Cell Culture and Assay for Methyl-³H]Thymidine (³H]TdR) Incorporation. Spleen cells were cultured at 5×10^5 per culture (in 0.2 ml) for proliferative responses, except where otherwise noted. Cells were cultured in a modified Mishell-Dutton medium (24) containing 10% fetal calf serum (FCS) (Rehatuin, Armour Pharmaceutical Co., Phoenix, Ariz.), 16-mM Hepes buffer, and 5×10^{-5} M 2-mercaptoethanol (2-ME), in flat-bottom microtiter plates (Microtest II, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and were assayed for [³H]TdR uptake at 48 h as previously described (22). For primary antibody responses to sheep erythrocytes (SRBC), culture conditions similar to those for [³H]TdR incorporation were used, except that 1×10^6 cells were added to each well. The number of plaque-forming cells (PFC) for SRBC were measured on day 4.

Depletion of T Cells by Anti-Thy 1.2 Antiserum or Rabbit Anti-Mouse Thymocyte Serum (ATS) Treatment. Spleen cells were suspended in a 1:5 or 1:10 dilution of an AKR anti-C3H (anti-Thy 1.2) antiserum (25) or in a 1:50 dilution of a rabbit anti-mouse thymocyte serum (ATS, batch 14580, Microbiological Associates, Walkersville, Md., generously supplied by Drs. John Kappler and Philippa Marrack, the University of Rochester, Rochester, N. Y.) in Hanks' balanced salt solution (HBSS) at 30×10^6 cells/ml and were held on ice for 30 min. The cells were subsequently pelleted and resuspended in a 1:4 dilution of absorbed guinea pig serum (Flow Laboratories, Inc., Rockville, Md.). After a 30-min incubation at 37°C, the cells were washed with HBSS and adjusted to 5×10^6 viable cells/ml in medium before plating 0.1 ml/culture. Control spleen cells were held on ice in HBSS, followed by incubation with complement.

Preparation of Splenic T Cells by Nylon Wool Column Passage. Spleen cells (300×10^6) in 5 ml HBSS containing 5% FCS were passed into a 3-g column of nylon wool (30-ml vol in a 50-ml syringe; Leuko-pac Leukocyte Filter, Fenwal Laboratories, Deerfield, Ill., 26). After a 45-min incubation at 37°C, the effluent cells were slowly collected from the column while maintaining

a 15-ml head volume above the column. The eluted cells contained 0–7% Ig⁺ cells, as detected by staining for surface Ig using a fluorescein-labeled goat anti-mouse Ig. This T-cell enriched population is referred to as “nylon wool T cells” (NWT).

Preparation of Ig⁺ and Ig⁻ Spleen Cell Populations. Separation of surface Ig⁺ cells from Ig⁻ cells was accomplished with a fluorescence activated cell sorter (FACS) (Becton, Dickinson & Co., Mt. View, Calif.) as previously described (27). Briefly, spleen cells from CBA/J mice were treated with ammonium chloride to lyse erythrocytes, or centrifuged on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N. J.) to remove dead cells and erythrocytes, washed in RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 10% FCS, and incubated with a fluorescein-conjugated F(ab')₂ fragment of a polyspecific goat anti-mouse Ig or a fluorescein-labeled F(ab')₂ rabbit anti-mouse Ig under aseptic conditions. After washing at 0°C, the cells were analyzed with the FACS, and a bimodal fluorescence profile was generated. Cells with relative fluorescence intensities of 0–25 were considered Ig⁻, whereas cells within channels 125–1,000 were considered Ig⁺. Cells with these fluorescence intensities were separated from each other with the FACS over a period of approximately 4 h. During the sorting process all cells were kept at 0°C to prevent capping. We routinely obtain Ig⁺ populations that are 94–96% pure and Ig⁻ populations that are 94–98% pure using this procedure.

Depletion of Adherent and Phagocytic Cell Populations. Spleen cells (400×10^6) were depleted of adherent cells by passage sequentially over two 30-ml columns of Sephadex G-10, (lot 9067, Pharmacia Fine Chemicals; 28) at 37°C. Adherent cells were also removed by incubation of spleen cells at 20×10^6 cells/ml in HBSS containing 5% FCS with 20 mg washed carbonyl iron (General Aniline & Film Corp., Easton, Pa.) at 37°C for 30 min (29). Cells that had taken up iron or to which the iron had adhered were removed by three settlings in 60×20 -mm Petri dishes (no. 3002, Falcon Plastics) placed on a ceramic magnet (35 lb pull, no. 42,098, Edmund Scientific Co., Barrington, N. J.). The depletion of macrophages was tested by phagocytosis of latex beads. Cells (15×10^6 in 1.5 ml) were incubated with 10 μ l of latex beads (1.091 μ m diameter, Uniform Latex Particles, Dow Chemical Co., Midland, Mich.) overnight, rotating at 37°C. The percentage of cells that had phagocytosed latex was measured by light microscopy. Peritoneal cells were prepared by injecting 5 ml HBSS into the peritoneal cavity of a normal mouse and withdrawing the fluid after brief agitation. The cells were immediately exposed to 1,500 rads of γ -irradiation from a Cs-137 source (Gammator M, Isomedix Inc., Parsippany, N. J.), washed, and added to cultures of spleen cells that had been depleted of adherent cells.

Mitogens. Lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4, Westphal or Boivin, Difco Laboratories, Detroit, Mich.) was used at 50 μ g/ml. Reference endotoxin (RE) *E. coli* 0113:H10 was kindly provided by Dr. Ronald Elin, Clinical Pathology Department, National Institutes of Health. Concanavalin A (Con A) (lot 7001, Pharmacia Fine Chemicals, Uppsala, Sweden) was used at 2 μ g/ml. Phytohemagglutinin-P (PHA; lot K1954, Wellcome Research Laboratories, Beckenham, England) was used at 1 μ g/ml. *Nocardia* water-soluble mitogen (NWSM; a generous gift of Dr. Constantin Bona, Pasteur Institute, Paris, France) was used at 10 μ g/ml.

Results

Tissue Distribution of Cells Responding to Anti-Ig. It has been previously shown (22) that spleen cells of BDF₁ mice are stimulated to a substantial proliferative response when cultured with affinity column-purified goat anti- μ antibody or goat anti- γ , κ antibody. As an initial step in the characterization of the cell type responsive to anti-Ig, lymphoid cell populations from various tissues were tested for responsiveness. Cells (5×10^5) from spleen, thymus, bone marrow, mesenteric and peripheral lymph node, and Peyer's patch were cultured for 48 h in the presence of an optimal stimulatory concentration of anti- μ or anti- γ , κ . The results of an experiment of this type, shown in Fig. 1, indicate that spleen, mesenteric lymph node, peripheral lymph node, and Peyer's patch cells given excellent responses to anti- μ ; bone marrow cells display an intermediate response, and thymocytes a minimal response. This pattern of responsiveness is similar to that observed for LPS, suggesting the importance of B lymphocytes in the stimulation caused by anti- μ . Furthermore, responsiveness of bone marrow

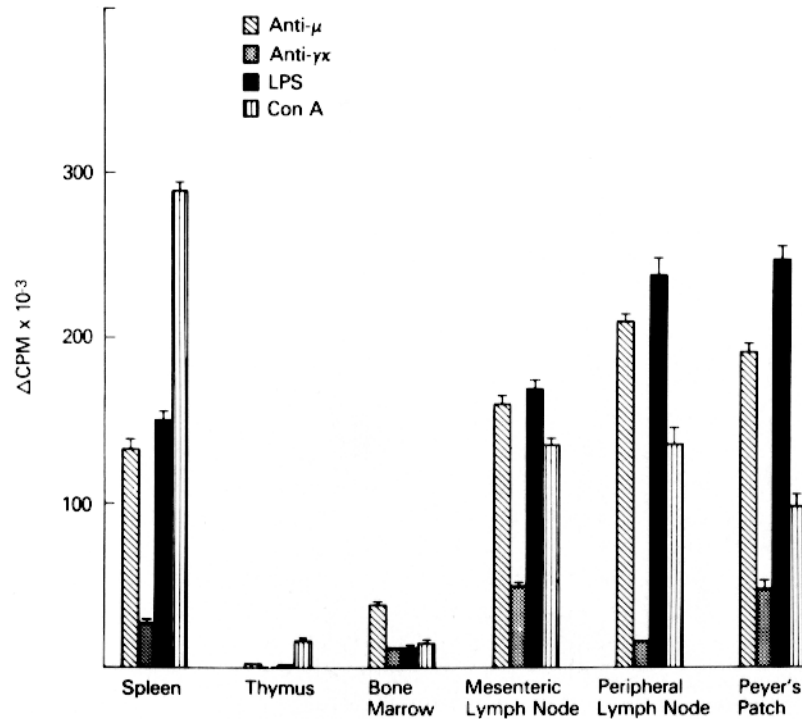


FIG. 1. Anti-Ig stimulation of cells from various lymphoid tissues. Cells were cultured at 5×10^5 per microwell in 0.2 ml medium containing the following stimulants: anti- μ (50 $\mu\text{g/ml}$), anti- γ,κ (250 $\mu\text{g/ml}$), LPS (50 $\mu\text{g/ml}$), or Con A (2 $\mu\text{g/ml}$). Responses were measured after 48 h of culture and a 16-h pulse with [^3H]TdR.

cells is consistent with a lack of a requirement for T lymphocytes in the anti-Ig response. As noted previously, the response to optimal concentrations of anti- μ is substantially greater than the response to anti- γ,κ . This difference is particularly striking in cultures of peripheral lymph node cells. Although subsequent data suggests that cells responding to anti- μ and anti- γ,κ are not identical, the reason for differences in relative responsiveness has not yet been established. The most obvious explanation is that the relative frequency of the responding cell type varies in different lymphoid tissues.

Depletion of T Cells by Anti-Thy 1.2 and Complement Treatment. To support the hypothesis that B cells are able to respond to anti-Ig in the absence of T cells, spleen cell populations, which were depleted of T cells by treatment with an anti-Thy 1.2 antiserum and complement, were tested for their ability to respond to anti-Ig. Table I shows that, although spleen cells depleted of Thy 1.2-bearing lymphocytes were unresponsive to Con A and PHA, such cells responded normally to anti- γ,κ and LPS. Furthermore, reconstitution of T-depleted spleen cell cultures with NWT cells restored Con A and PHA responsiveness but had no effect on anti- γ,κ or LPS responses. The response anti- μ in these and other experiments showed a slight diminution after anti-Thy 1.2 and complement treatment. The loss in response was somewhat restored by the addition of NWT cells. However, the majority of the anti- μ response was maintained after such treatment, suggesting that the majority of anti- μ -responsive B cells can be stimulated in the absence of T cells. NWT cells alone were unable to

TABLE I
Effect of Pretreatment with Anti-Thy 1.2 Antiserum and Complement on Anti-Ig-Induced Proliferation*

Mitogen	Concentration	No treatment	Complement alone	Anti-Thy 1.2 + Complement	Anti-Thy 1.2 + Complement with NWT‡	NWT‡ alone
	$\mu\text{g/ml}$			<i>cpm per culture</i>		
None	—	13,333 \pm 461	19,196 \pm 1,453	6,410 \pm 237	14,141 \pm 802	183 \pm 41
G102 anti- μ	100	125,947 \pm 12,591	187,997 \pm 10,151	171,754 \pm 4,926	178,582 \pm 3,411	894 \pm 99
	10	92,700 \pm 8,869	108,008 \pm 4,542	81,259 \pm 1,224	119,353 \pm 13,324	408 \pm 46
G125 anti- γ,κ	200	23,711 \pm 1,511	33,505 \pm 1,808	55,306 \pm 2,009	54,260 \pm 2,676	155 \pm 68
	50	19,873 \pm 1,823	32,411 \pm 2,840	38,745 \pm 2,125	50,669 \pm 1,920	42 \pm 55
LPS	50	161,818 \pm 7,293	182,484 \pm 5,893	147,721 \pm 3,385	143,089 \pm 1,878	1,120 \pm 153
Con A	2	281,598 \pm 2,521	279,039 \pm 12,745	8,836 \pm 2,475	265,661 \pm 4,219	85,912 \pm 2,231
PHA	1	156,193 \pm 11,268	157,340 \pm 3,852	3,792 \pm 110	185,547 \pm 8,396	83,224 \pm 1,027

* 3×10^5 cells per culture.

‡ 1×10^6 NWT.

respond significantly to anti-Ig or LPS, but did respond to the T-cell mitogens. Thus, it appears that the response to anti- μ or anti- γ,κ of spleen cells is largely a response of B lymphocytes which does not require T-lymphocyte help and that T lymphocytes, when cultured alone, do not respond to anti-Ig.

Anti-Ig Stimulation of FACS-Sorted Spleen Cells. To correlate the response to anti-Ig with the presence of Ig on the membrane of responding cells, spleen cells were stained with fluoresceinated $F(ab')_2$ fragments of a goat anti-Ig reagent and sorted into Ig^- and Ig^+ populations by the FACS. It should be noted that staining of the Ig^+ B cells with the fluorescein-conjugated $F(ab')_2$ anti-Ig reagent did not cause any stimulation on its own, because there were no differences in background levels of [3H]TdR uptake between nontreated spleen cells and those treated with fluorescein-conjugated anti-Ig. Similarly, staining did not influence a subsequent response to either anti- μ or anti- γ,κ . This is consistent with other data² showing an inability to pulse-stimulate spleen cells with either anti- μ - or anti- γ,κ -antibodies.

A profile of the distribution of the amount of Ig per cell in the whole spleen cell population is shown in Fig. 2. Cells with fluorescence intensities detected in channels 0 through 25 (39.9% of total) were classed as Ig^- , whereas cells with fluorescence intensities detected in channels 125 to 1,000 (56.5% of total) were classed as Ig^+ . After separation, the two populations were cultured at 2×10^5 cells per culture in the presence of anti- μ , anti- γ,κ , and conventional T- or B-cell mitogens. The results in Fig. 3 show that the Ig^- pool failed to respond to anti- μ or to anti- γ,κ . These cells responded minimally to LPS, while giving substantial responses to Con A and PHA. In contrast, anti- μ , anti- γ,κ , and LPS stimulated responses in cultures of nonsorted spleen cells and in cultures of Ig^+ spleen cells. Indeed, the responses of Ig^+ cells to anti- γ,κ in this experiment were significantly greater than the response of nonsorted cells, suggesting an enrichment for the anti- γ,κ responding cell in the Ig^+ pool. This enrichment has also been noted for the anti- μ response in the majority of experiments of this type, although it was not seen in the experiment illustrated in Fig. 3.

It was noted that cells in the Ig^+ pool still retained some responsiveness to Con A and PHA. This suggested the presence of at least some contaminating T lymphocytes in the Ig^+ population. To overcome this difficulty we attempted to sort spleen cells that had been previously depleted of T lymphocytes by treatment with a rabbit ATS

² D. G. Sieckmann and W. E. Paul. Unpublished observations.

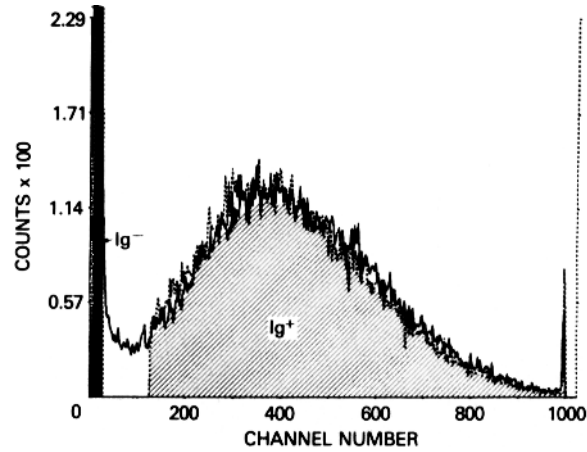


FIG. 2. Fluorescence profile of CBA/J spleen cells stained with fluorescein-labeled $F(ab')_2$ goat anti-mouse Ig (—). A second profile (---) shows Ig^- (channels 0–25) and Ig^+ (channels 125–1,000) pools as they were detected during the sorting process.

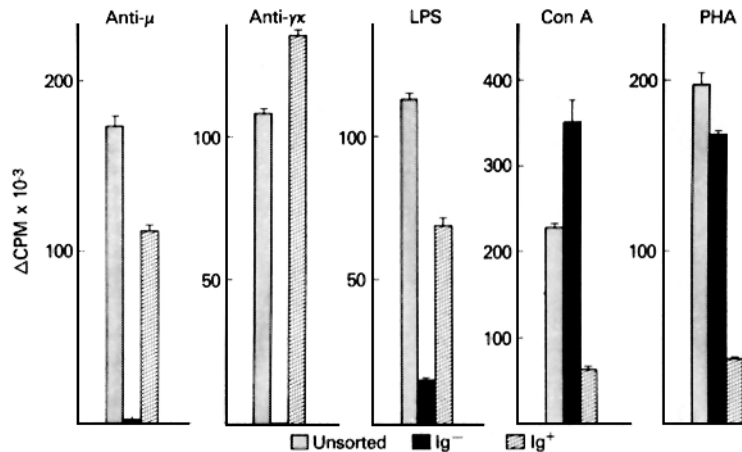


FIG. 3. Anti-Ig stimulation of FACS-sorted spleen cells. Nonsorted, Ig^+ , or Ig^- cells were cultured at 2×10^5 cells per microwell with anti- μ (100 $\mu\text{g}/\text{ml}$), anti- $\gamma\kappa$ (250 $\mu\text{g}/\text{ml}$), LPS (50 $\mu\text{g}/\text{ml}$), Con A (2 $\mu\text{g}/\text{ml}$), or PHA (1 $\mu\text{g}/\text{ml}$).

and complement. Preliminary experiments showed that pretreatment of spleen cells with ATS did not alter the anti-Ig staining profile of Ig^+ cells, when such cells were stained with a fluorescein-labeled rabbit $F(ab')_2$ anti-mouse Ig. Cells treated in this manner were sorted on the FACS. Ig^+ cells were collected from channels 451 to 1,000. Such cells, when reanalyzed after sorting, were 98.3% Ig^+ . For comparison, normal cells were also sorted into Ig^- (channels 0–45) and Ig^+ (channels 90–1,000) cells. The results of this experiment (Table II) demonstrate that ATS and complement-treated- Ig^+ -sorted spleen cells, although completely unresponsive to Con A or PHA, were still able to produce a vigorous response to both anti- μ and anti- $\gamma\kappa$. These responses to anti-Ig were quite enhanced as compared with normal unsorted spleen cells. The response to LPS was also maintained. These results indicate that anti-Ig stimulation

TABLE II
Anti-Ig Proliferative Response of FACS-Sorted Spleen Cells Previously Depleted of T Cells by ATS + Complement Treatment*

Stimulant	Concentration	Normal			ATS + Complement treated	
		Unsorted	Ig ⁺	Ig ⁻	Unsorted	Ig ⁺
	$\mu\text{g/ml}$					
None	—	2,536 \pm 153	1,329 \pm 69	195 \pm 7	3,441 \pm 233	1,537 \pm 120
G102 anti- μ	100	95,201 \pm 1,342	100,429 \pm 3,236	980 \pm 49	132,329 \pm 2,608	140,684 \pm 4,012
G125 anti- γ,κ	200	12,219 \pm 1,497	12,885 \pm 1,181	244 \pm 34	22,118 \pm 2,156	52,213 \pm 3,010
LPS	50	114,226 \pm 6,515	184,688 \pm 16,573	3,388 \pm 290	109,244 \pm 11,130	169,078 \pm 8,691
Con A	2	300,087 \pm 7,806	23,272 \pm 2,693	155,915 \pm 11,869	4,854 \pm 977	1,147 \pm 211
PHA	1	196,297 \pm 5,755	24,371 \pm 15	117,266 \pm 7,741	1,820 \pm 115	387 \pm 50

* BDF₁ spleen cells were treated with a rabbit ATS and complement before staining with a fluorescein-labeled rabbit F(ab)₂ anti-mouse Ig. Ig⁺ cells were sorted on the FACS into channels 451-1,000. Normal spleen cells were stained in a similar manner and sorted into Ig⁻ (channels 0-45) and Ig⁺ (channels 90-1,000) cells. Cells were cultured at a density of 1.5×10^5 per microwell.

TABLE III
Stimulation of Athymic Nude (nu/nu) Mice by Anti-Ig*

Mitogen	Concentration	nu/+		nu/nu		NWT‡
		—	1×10^5 NWT	—	1×10^5 NWT	
	$\mu\text{g/ml}$					
Experiment I						
None	—	3,382 \pm 240	6,160 \pm 674	2,236 \pm 247	4,966 \pm 1,023	300 \pm 32
G615 anti- μ	50	94,770 \pm 9,600	112,537 \pm 9,262	49,225 \pm 3,813	68,575 \pm 5,103	20,401 \pm 561
G125 anti- γ,κ	250	10,516 \pm 2,523	18,770 \pm 2,947	1,198 \pm 142	4,153 \pm 116	2,018 \pm 114
LPS	50	159,232 \pm 7,706	161,331 \pm 7,447	128,849 \pm 12,828	121,190 \pm 10,335	9,981 \pm 194
Con A	2	232,894 \pm 12,277	213,715 \pm 17,280	1,936 \pm 197	169,407 \pm 1,601	277,692 \pm 7,001
Experiment II						
None	—	7,339 \pm 2,155	10,343 \pm 2,721	2,209 \pm 195	5,332 \pm 933	190 \pm 40
G615 anti- μ	50	95,440 \pm 3,585	89,295 \pm 1,551	69,660 \pm 9,569	77,803 \pm 9,771	5,967 \pm 139
G125 anti- γ,κ	250	18,675 \pm 3,917	30,754 \pm 8,625	2,816 \pm 564	8,704 \pm 2,030	378 \pm 16
LPS	50	114,782 \pm 6,169	85,981 \pm 4,301	82,564 \pm 6,680	79,186 \pm 6,185	7,384 \pm 85
Con A	2	167,554 \pm 7,269	146,248 \pm 4,899	3,470 \pm 623	20,803 \pm 6,710	63,035 \pm 2,246
Experiment III						
None	—	4,232 \pm 340	8,509 \pm 2,836	1,840 \pm 167	2,464 \pm 367	45 \pm 7
G125 anti- γ,κ	250	24,923 \pm 4,390	32,251 \pm 8,758	7,171 \pm 1,273	8,027 \pm 1,173	81 \pm 7
LPS	50	103,203 \pm 2,182	97,084 \pm 2,931	74,144 \pm 14,043	73,684 \pm 13,104	296 \pm 58
Con A	2	201,111 \pm 7,725	166,252 \pm 5,929	1,143 \pm 139	205,307 \pm 16,160	66,590 \pm 7,006

* Spleen cells from 10 to 12-week-old athymic (nu/nu) mice or heterozygous littermates (nu/+) were cultured individually. Each of the values expressed in this table represents the arithmetic mean \pm standard error of determinations made on four individual mice.

‡ Nylon wool T cells were cultured alone at 1×10^5 cells/culture (exp. II and III) or at 5×10^5 cells/culture (exp. I).

is dependent upon an Ig⁺ cell and that Ig⁻ cells are not required for responses by Ig⁺ cells.

Stimulation of Athymic (nu/nu) Mice by Anti-Ig. To obtain additional evidence for the T-cell independence of B-cell stimulation by anti-Ig reagents, the ability of spleen cells from nu/nu BALB/c mice to respond to anti-Ig was examined (Table III). Spleen cells from nu/nu and from nu/+ BALB/c mice (10-12 wk of age) were cultured individually with various concentrations of anti- μ , anti- γ,κ , and standard concentrations of LPS or Con A. Results are displayed as the arithmetic mean of the maximum response to anti-Ig of the four individual mice in each group. Cultures of nu/nu spleen cells responded quite well to anti- μ ; however, their responses were only 50-70% of that of normal (nu/+) heterozygous littermates. Although not shown in Table III, the lower responsiveness of nu/nu spleen cells was apparent at all concentrations of anti- μ tested. Nu/nu spleen cells also responded somewhat less well to LPS than did nu/+ spleen cells. Responses of nu/nu spleen cells to anti- μ were only marginally

TABLE IV
*Effect of Removal of Adherent Cells from Spleen Cell Populations on Anti-Ig-Induced Proliferation**

Mitogen	Concentration $\mu\text{g/ml}$	No treatment	Sephadex G-10 passed	Carbonyl iron treated
			<i>cpm per culture</i>	
None	—	7,251 \pm 438	7,885 \pm 181	9,001 \pm 250
G615 anti- μ	50	84,220 \pm 4,007	94,260 \pm 3,198	78,164 \pm 3,405
	10	58,732 \pm 3,391	77,756 \pm 1,112	68,732 \pm 6,329
G125 anti- γ,κ	250	29,912 \pm 2,992	67,107 \pm 3,841	83,241 \pm 4,547
	50	23,971 \pm 3,766	43,176 \pm 9,285	52,976 \pm 2,648
LPS	50	72,665 \pm 2,203	72,840 \pm 881	66,634 \pm 4,163
Con A	2	230,238 \pm 8,759	55,271 \pm 3,739	119,240 \pm 7,859
PHA	1	103,545 \pm 2,766	98,970 \pm 7,735	111,169 \pm 4,419

* BDF₁ spleen cells (15 wks of age) were passed through two sequential Sephadex G-10 columns or treated with carbonyl iron. Nontreated spleen or nonadherent cell populations were cultured at 5×10^5 cells per culture.

enhanced by addition of nylon wool-purified splenic T cells obtained from *nu/+* littermates. In contrast, responsiveness of *nu/nu* spleen cells to anti- γ,κ was quite variable, and most individuals were either nonresponsive or responded only minimally. Again, addition of NWT cells did not restore responsiveness to anti- γ,κ , suggesting that the poor responses by the *nu/nu* spleen cells are not the result of the absence of a functional T-cell population, but reflect an abnormality in the B cells of these mice.

Effect of Removal of Adherent Cells from Spleen Cell Populations on Anti-Ig-Induced Proliferation. We have previously reported that F(ab')₂ fragments prepared from anti- μ are as stimulatory as whole molecules in inducing a proliferative response (22). This would suggest that binding to Fc receptors on either lymphocytes or macrophages was not required for stimulation. It was nevertheless of interest to determine whether or not the presence of macrophages was important in B-cell responses to anti-Ig.

Two methods were used for depletion of adherent cell populations: (a) sequential passage of spleen cells over two Sephadex G-10 columns (28) or (b) carbonyl iron treatment (29). The effectiveness of macrophage removal was monitored by testing for responsiveness to SRBC, which is a macrophage-dependent response, and by testing for phagocytosis of latex particles. The results of one such experiment are shown in Table IV. In this particular experiment, the day 4 primary SRBC response (not shown) was abrogated to the extent of 99 and 90% by G-10 column and carbonyl iron treatment, respectively, and these responses could be partially reconstituted by addition of 2% irradiated peritoneal cells. The starting spleen cell population had 7.1% phagocytic cells by the latex test, G-10 column and carbonyl iron treatment reduced this to 1.8 and 0.9%, respectively. The results in Table IV show that neither anti- μ nor anti- γ,κ responses were affected by either treatment. The LPS and PHA responses similarly were unaffected, whereas the Con A response was considerably diminished, as has been previously reported (30).

The above results suggested that macrophages are not required for anti-Ig-induced proliferation. However, because we had not completely removed all phagocytic cells, as detected by the latex phagocytosis assay, we tested responsiveness of depleted cell populations at limiting cell densities. We reasoned that if small numbers of residual macrophages supported responses in our standard culture condition (5×10^5 cells/culture), they should be diluted out at lower cell concentrations. In this experi-

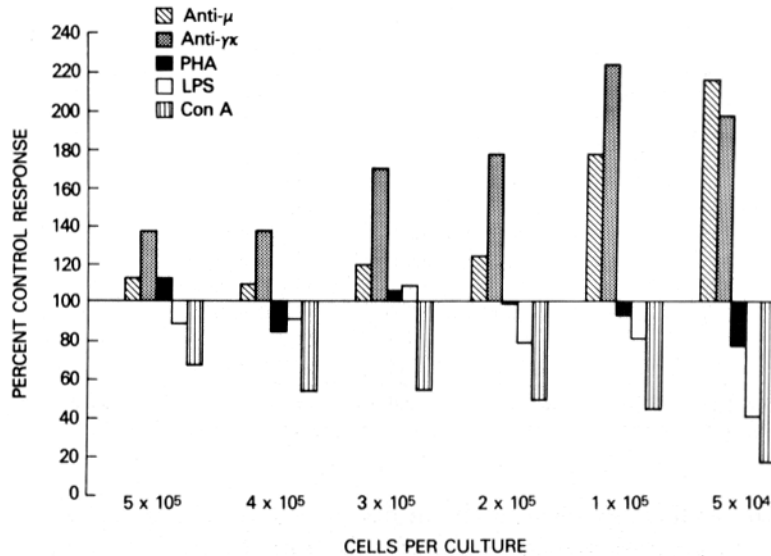


FIG. 4. Response of macrophage-depleted cells at various cell densities. BDF₁ spleen cells (15 wk of age) were treated with carbonyl iron and cultured at densities of 5×10^4 to 5×10^5 cells per culture with anti- μ (50 $\mu\text{g}/\text{ml}$), anti- $\gamma\kappa$ (250 $\mu\text{g}/\text{ml}$), LPS (RE, 50 $\mu\text{g}/\text{ml}$), Con A (2 $\mu\text{g}/\text{ml}$), and PHA (1 $\mu\text{g}/\text{ml}$). Results are reported as the response of nonadherent spleen cells + response of control normal spleen cells $\times 100\%$.

ment, phagocytic cells were removed by treatment with carbonyl iron and exposure to a magnetic field. This treatment diminished the frequency of cells capable of phagocytosing latex particles from 4.0 to 0.9% and diminished the *in vitro* primary anti-SRBC antibody response by 80%. The cells were then cultured at concentrations of 5×10^4 to 5×10^5 cells per microwell. The results are presented in Fig. 4 as the ratio of the response of macrophage-depleted cells to that of untreated cells, multiplied by 100. The results show that the relative responsiveness of macrophage-depleted cells to Con A falls as the number of cells cultured is diminished. However, the relative responsiveness of macrophage-depleted cells to anti- μ and to anti- $\gamma\kappa$ actually increases upon cell dilution. Thus, at 5×10^4 cells per microwell, the response to anti- μ and to anti- $\gamma\kappa$ is approximately twofold greater in macrophage-depleted populations than in unseparated cell populations. In every case, addition of irradiated peritoneal cells (2%) to macrophage-depleted populations returned the response to anti-Ig to that of unseparated cells (data not shown). These results provide strong support for the concept that macrophages are not required for anti-Ig stimulation and that they may be suppressive under certain circumstances.

Ontogeny of the Anti-Ig Response. We have previously reported that anti- μ is able to stimulate responses of spleen cells from a wide variety of mouse strains at 8–12 wk of age. A more complete study of the ontogeny of responsiveness was made to determine if there was any correlation between responsiveness to anti- μ and anti- $\gamma\kappa$, and the development of various B-cell types in the spleen. Cultures of spleen cells from BDF₁ mice ranging from 1 to 32 wk of age were stimulated with either anti- μ , anti- $\gamma\kappa$, LPS, or NWSM (31). The responses are shown in Fig. 5 as the absolute response and as the percent of the average adult response for each mitogen. From these two graphs, it can be seen that spleen cells from mice younger than 4 wk of age do not respond to anti-

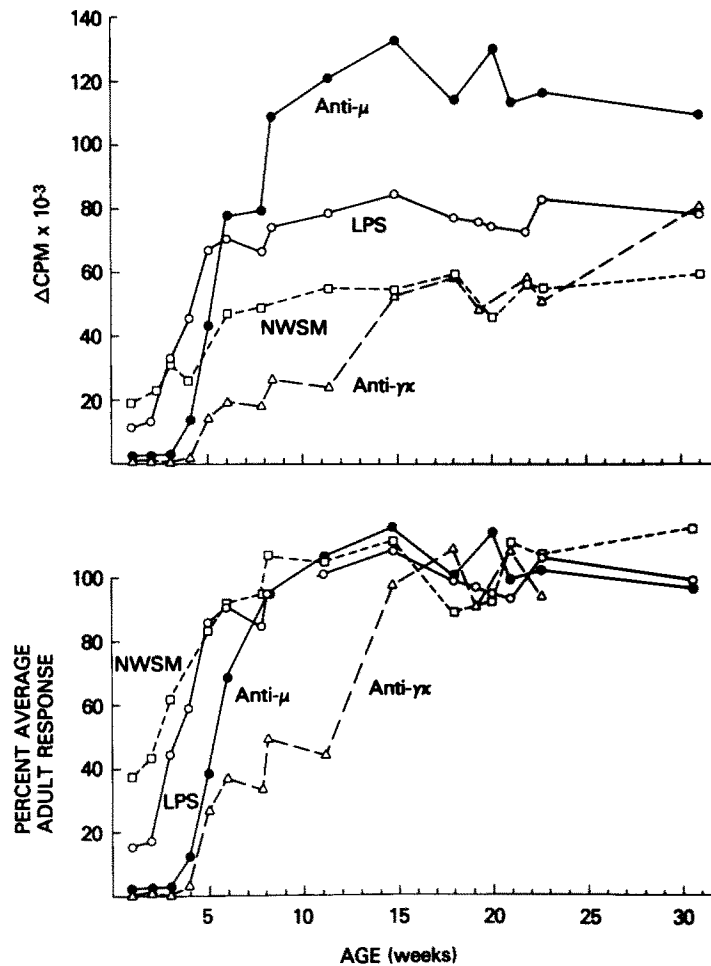


FIG. 5. Ontogeny of the anti-Ig response in BDF₁ mice. BDF₁ spleen cells from two or three mice of various ages were cultured with anti- μ (100 $\mu\text{g}/\text{ml}$), anti- γ,κ (100 $\mu\text{g}/\text{ml}$), LPS (50 $\mu\text{g}/\text{ml}$), and NWSM (50 $\mu\text{g}/\text{ml}$). The results are reported as Δcpm (upper graph) or as a percentage of the average maximum response calculated from data points in the plateau of the age response curve (lower graph). Calculated average maximum adult responses used for percentage calculations were: anti- μ , 114,263 \pm 867; anti- γ,κ , 54,229 \pm 4,588; LPS, 78,694 \pm 4,451; and NWSM, 51,885 \pm 6,881.

μ or to anti- γ,κ . Adult levels of responsiveness to anti- μ are not reached until after 8 wk. Responsiveness to anti- γ,κ can usually be observed at 4 wk of age although in the experiment illustrated, responses were first noted at 5 wk. This response, however, does not reach adult levels until after 15 wk of age. The developmental patterns of responsiveness to anti- μ and anti- γ,κ are quite different from those of responsiveness to LPS and NWSM. These mitogens stimulate responses by cells from 1-wk-old mice and give responses that are 60% of adult levels at 4 wk of age, at a time when responses to anti- μ are only 10% of adult levels. These results indicate that the B cells responsive to anti-Ig are members of a late-developing subset.

Anti-Ig Stimulation of Lymphocytes from (CBA/N \times DBA/2N) F_1 Mice. The CBA/N mouse strain has been shown to carry an X-linked genetic defect (23), which appears

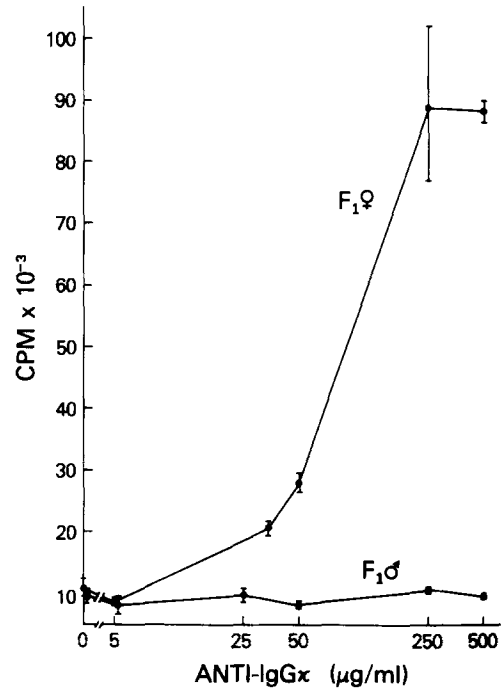


FIG. 6. Failure of anti- $\gamma_2\kappa$ to stimulate (CBA/N \times DBA/2N)F₁ male spleen cells. Spleen cells from F₁ male or female mice (19 wk old) were cultured at 5×10^6 cells/culture with various concentrations of anti- $\gamma_2\kappa$.

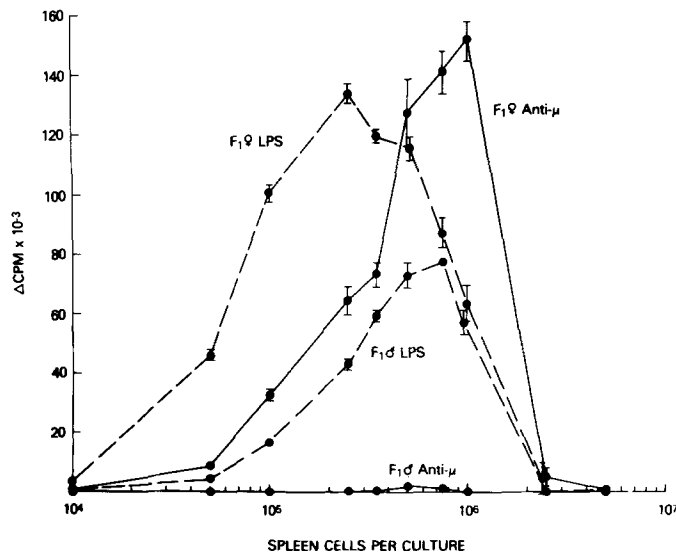


FIG. 7. Failure of anti- μ to stimulate (CBA/N \times DBA/2N)F₁ male spleen cells. Spleen cells of F₁ male (17 wk old) or female (19 wk old) mice were cultured with G615 anti- μ antiserum (1:200) or LPS (50 $\mu\text{g/ml}$).

to be due to the absence or defective function of a mature or late-developing subset of the B-lymphocyte population (32, 33). Fig. 6 shows that spleen cells from (CBA/N \times DBA/2N) F_1 male mice, which carry this defect, cannot be stimulated to a proliferative response by anti- γ,κ antibodies over a wide range of antibody concentrations, whereas spleen cells from F_1 female donors, which are phenotypically normal, exhibit a vigorous response. Fig. 7 shows that the unresponsiveness is also seen with anti- μ antibody in cultures of various numbers of spleen cells. In contrast, the F_1 -defective male mice respond well to LPS, although higher cell densities are required for maximum responses than are needed for responses of F_1 female cells. The unresponsiveness of lymphocytes from mice that carry the CBA/N B cell defect further suggests that anti- μ and anti- γ,κ selectively activate a mature subset(s) of Ig^+ B lymphocytes, which are functionally absent in these mice and in normal mice of <4 wk of age.

Discussion

In our previous paper (22), we demonstrated that specifically purified goat anti- μ , goat anti- γ,κ , and rabbit anti- κ antibodies could initiate substantial DNA synthetic responses by spleen cells from a wide variety of mouse strains, and we presented evidence indicating that this stimulatory activity depended upon the specificity of the antibody for Ig.

In this paper, we have studied the cellular basis of this response. Our results indicate that Ig^+ cells prepared by sorting for the Ig^+ phenotype or by pretreatment with anti-Thy 1.2 antiserum and complement can respond to anti-Ig, that T lymphocytes are not required for the response, and that thymocytes and nylon wool-passed spleen cells do not respond. The response of cells from *nu/nu* donors to anti- μ was somewhat lower than that of cells from phenotypically normal littermates, and *nu/nu* cells often did not respond to anti- γ,κ . However, the addition of nylon wool-purified splenic T cells from a *nu/+* littermate donor to cultures of *nu/nu* spleen cells had little effect on the anti- μ response and did not reconstitute the anti- γ,κ response. This suggests that B lymphocytes from *nu/nu* mice may be abnormal, particularly in the development of the subset of mature B lymphocytes that respond to anti-Ig. It also suggests that maturation of these cells may be under T-cell control. Nonetheless, these results indicate that both anti- μ and anti- γ,κ principally stimulate B lymphocytes and that their response is T independent.

The data presented here strongly suggest that the stimulatory effect of anti- μ and of anti- γ,κ is not exerted on all Ig-bearing lymphocytes. This conclusion is supported by the failure of anti- μ to stimulate proliferative responses of spleen cells from BDF₁ mice until the donors are 4 wk of age. Indeed, it has previously been shown (27, 34) that significant numbers of Ig^+ cells are present in the spleen 3 days after birth and adult frequencies of Ig^+ cells are present by 2 wk of age. In contrast, maximal responsiveness to anti- μ is not reached until 8 wk of age. Responses to anti- γ,κ develop even more slowly. The rate of development of responsiveness to anti-Ig contrasts strikingly with that to LPS and NWSM. The latter stimulate substantial responses by cells from 1-wk-old donors. Finally, mice with the CBA/N immune defect are unresponsive to both anti- μ and to anti- γ,κ . It has previously been shown that these mice lack a subset of mature or late-developing B lymphocytes which bear a distinct phenotypic marker, Lyb 5 (35). It appears that the B cells that proliferate in response to anti- μ and to anti- γ,κ are members of this Lyb 5⁺ subset. Indeed, preliminary

experiments³ indicate that pretreatment with anti-Lyb 5.1 and complement destroys responses to anti- μ and to anti- γ,κ , strongly supporting the idea that Lyb 5⁺ cells are responsible for the anti-Ig response. The distinctive response patterns to anti- μ and to anti- γ,κ may indicate a degree of heterogeneity within this population.

Our results, indicating that spleen cells from 4-wk-old mice can respond to anti-Ig, are quite different from those of Wiener et al. (21) who have reported that responsiveness to anti-Ig is not achieved until mice are approximately 7 mo of age. Although we cannot explain this difference, it seems unlikely to us that the natural process of lymphocyte differentiation is not completed until mice are 7 mo of age. It is possible that the failure of Wiener et al. to observe responses in young adult mice may reflect differences in the culture conditions used in our laboratories.

Wiener et al. also reported (36) that adult thymectomy speeded the rate at which anti-Ig responsiveness was obtained, whereas we noted that congenitally athymic mice were less responsive to anti- γ,κ than were their normal littermates. This difference may be explicable by the considerable difference in the T-lymphocyte status of these two types of mice. In the congenitally athymic animal, the peripheral environment is exceptionally depleted of T-lymphocyte influence, whereas in the adult thymectomized animal, there is preservation of the long-lived T-lymphocyte population. Both sets of observations point to the possibility that T lymphocytes (or thymic influence), although not required for response of mature B lymphocytes to anti-Ig, may play a role in the development of responsive cells.

The response of B lymphocytes to anti-Ig reagents is largely independent of the action of macrophages. Thus, depletion of adherent cells by Sephadex G-10 or by treatment with carbonyl iron and exposure to a magnetic field did not diminish responsiveness to anti-Ig reagents. Indeed, when cell populations depleted of phagocytic cells were cultured at limiting cell numbers (5×10^4 /well), their responses to anti- μ and to anti- γ,κ were greater than those of unseparated spleen cells. These enhanced responses were abolished when irradiated normal peritoneal cells, as a source of macrophages, were added to macrophage-depleted populations. Furthermore, in preliminary experiments, we have removed macrophages by carbonyl iron treatment and exposure to a magnetic field and then isolated Ig⁺ cells from this macrophage-depleted population. Such cells, lacking both macrophages and T cells, responded well to anti- μ and anti- γ,κ . These results strongly suggest that the stimulatory effect of anti-Ig reagents is independent of both macrophages and T cells. However, one cannot absolutely exclude the need for very small numbers of either cell type. Other types of cell-cell interaction in the activation caused by anti-Ig (e.g. B-B interaction or interaction of nonadherent, nonphagocytic accessory cells with B cells) have not been formally excluded. Nevertheless, our results are most consistent with the concept that anti-Ig leads to the direct activation of mouse B lymphocytes. Inasmuch as F(ab')₂ fragments of anti- μ and deaggregated anti- μ are equivalent to control anti- μ preparations in B-cell activation, it does not appear likely that Fc receptors participate in the response. Furthermore, responses to anti- μ can be obtained in serum-free media (22), although such responses do depend on the addition of 2-ME. We would propose that the activation of B-lymphocyte proliferation by anti- μ and anti- γ,κ antibodies is directly due to an interaction with Ig determinants on the B-cell membrane and that the generation of transmembrane signals is a function of

³ D. G. Sieckmann and B. Subbarao. Unpublished observations.

the Ig determinants and not of some postulated non-Ig receptor for a mitogenic principle (10, 11).

Although not addressed in this paper, anti-Ig fails to stimulate Ig synthesis despite the marked uptake of [³H]TdR which occurs in stimulated cells. This is consistent with the need for intrinsically different types of stimuli for activation of the proliferative and differentiative aspects of normal B-lymphocyte responses. As anti-Ig antibody is a globular protein similar in general structure to most thymus-dependent antigens, its interaction with B-lymphocyte Ig may be regarded as comparable to the binding of a thymus-dependent antigen to receptors of specific B lymphocytes. In the absence of T-cell influence, such interactions may lead to priming and proliferation of specific cells but not to antibody synthesis (37-39). Furthermore, Kishimoto and Ishizaka (40) have reported that a T-lymphocyte supernate factor, together with anti-Ig, may cause Ig synthesis by rabbit lymphocytes. Current efforts are underway to find conditions that allow anti-Ig antibodies to stimulate Ig synthesis in mouse cells. This should provide a system in which the distinctive components of B-lymphocyte activation can be precisely analyzed.

Summary

Mouse spleen cells can be stimulated to proliferate *in vitro* by purified anti- μ or anti- $\gamma_1\kappa$ antibodies. These responses can be obtained in cell populations bearing membrane immunoglobulin (Ig), purified by the fluorescence activated cell sorter (FACS), but they are not observed in FACS-purified Ig⁻ cell populations. Furthermore, treatment of spleen cell populations with anti-Thy 1.2 and complement does not impair the response, nor does addition of nylon wool-purified T lymphocytes enhance it. These results indicate that B lymphocytes respond to anti-Ig and that their response does not require T cells. On the other hand, cells from athymic nude (*nu/nu*) mice respond slightly less well to anti- μ than do cells from heterozygous littermate (*nu/+*) controls; *nu/nu* cells are almost unresponsive to anti- $\gamma_1\kappa$ and addition of nylon wool-purified T cells from *nu/+* controls does not restore the response. This suggests that T lymphocytes or the thymus may control the appearance of cells responsive to anti- $\gamma_1\kappa$.

Responsiveness of normal mice to anti- μ does not appear until 4 wk of age and does not reach maximum levels until 8 wk of age. Acquisition of full responsiveness to anti- $\gamma_1\kappa$ is even more delayed. This, together with the failure of mice with the CBA/N B-cell defect to respond to anti-Ig, suggests that cells stimulated to proliferate by anti-Ig are a mature subset of B cells.

Depletion of adherent cells by Sephadex G-10 treatment or by treatment with carbonyl iron and exposure to a magnetic field does not diminish anti- μ or anti- $\gamma_1\kappa$ responses, suggesting that the responsiveness does not require the presence of macrophages. Thus, activation of B-cell proliferation by anti-Ig appears to be a T-cell independent, macrophage-independent process in which membrane Ig plays a direct role in signal generation.

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