Title
Frequency of B lymphocytes responsive to anti-immunoglobulin.

Journal
Journal of Experimental Medicine, 155(5)

ISSN
0022-1007

Authors
Defranco, AL
Raveche, ES
Asofsky, R
et al.

Publication Date
1982-05-01

DOI
10.1084/jem.155.5.1523

Peer reviewed

FREQUENCY OF B LYMPHOCYTES RESPONSIVE TO ANTI-IMMUNOGLOBULIN

By ANTHONY L. DEFRANCO,* ELIZABETH S. RAVECHE, RICHARD ASOFSKY, AND WILLIAM E. PAUL

From the Laboratory of Immunology and the Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, and the Laboratory of Experimental Pathology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

The specificity of the immune response is known to be a result of the presence of receptors for antigen on the surface of lymphocytes. For B lymphocytes the receptors are types of immunoglobulins specially designed to be membrane proteins, principally membrane IgM and membrane IgD (mIgM and mIgD).[1] From numerous studies, first with rabbit lymphocytes (1), and later with human (2, 3) and mouse (4–6) lymphocytes, it has become clear that many of the supposed consequences of interaction of B lymphocyte receptors with antigen could be induced by the interaction of such receptors with anti-receptor antibody. For the most part, this has involved the demonstration that anti-immunoglobulin (Ig) antibodies such as anti-allotype or anti-isotype antibodies cause polyclonal B cell proliferation, usually demonstrated by uptake of radiolabeled thymidine. Earlier events in B cell activation, including increased protein and RNA synthesis and cell enlargement, have also been observed as a consequence of stimulation with anti-Ig antibodies (7). Furthermore, both Kishimoto and Ishizaka (8) and Parker et al. (9) have shown that anti-Ig-stimulated B cells may become antibody-secreting cells if soluble mediators present in the supernatant of spleen cells stimulated with antigen or concanavalin A (Con A) are added to these cultures.

To better understand this model of the activation of B cells by antigen, it is important to determine whether all B cells respond to anti-Ig in each of these ways or whether only some subpopulations are capable of each response. Indeed, several studies now indicate that not all mouse splenic B cells proliferate in response to anti-Ig antibody. For example, B cells from young mice (3–6 wk old) respond very well to lipopolysaccharide (LPS), but only poorly to anti-Ig antibodies, such as anti-μ (10). Similarly, cells with large amounts of mIgM or small amounts of mIgD give poor responses to anti-μ, as assessed by [^{3}H]thymidine uptake (11). Even more striking is the inability of B cells from mice with the *xid*-determined immune defect to proliferate in response to this stimulus (10). These B cells do proliferate in response to LPS. Finally, the activation state of the B cell before removal from the animal has recently been shown to have a significant effect in determining which activation signals are

* Supported by a fellowship from the Helen Hay Whitney Foundation.

[1] *Abbreviations used in this paper:* BDF_1, $(C57BL/6 \times DBA/2)F_1$; CDF_1, $(CBA/N \times DBA/2N)F_1$; Con A, concanavalin A; DCF_1, $(DBA/2N \times CBA/N)F_1$; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; mIgM, mIgD, membrane form of IgM or IgD; NP-40, Nonidet P-40; PBS, phosphate-buffered saline.

required in vitro to stimulate it to proliferate and differentiate into antibody-secreting cells (12). These observations indicate that both the degree of development and the state of activation of a B cell may influence its ability to respond to anti-μ. In the current studies, we have studied the capacity of resting B cell populations to enter the G_1 phase of the cell cycle in response to anti-μ. In addition, we have directly measured the fraction of B cells that synthesize DNA in response to anti-μ through the use of the quantitative fluorescent DNA stain propidium iodide and by flow microfluorimetry.

In both types of experiments, we used density-gradient centrifugation to separate resting and partially activated B cell populations and then cultured the resting B cells in the completely defined serum-free medium (Iscove's/F-12 medium) recently developed by D. E. Mosier (13) from the original serum-free formulation of Iscove and Melchers (14). The homogenous size distribution of these cells made it possible to study size enlargement in response to anti-μ. We found that virtually all small, dense B cells enlarged during the first 24 h of stimulation and that this enlargement was detectable within 1 h of addition of anti-μ. In contrast, only ~60% of resting B cells synthesized DNA in response to anti-μ. On the basis of these results, we conclude that anti-μ stimulates all resting B cells to enter G_1, but that only a fraction of these cells continue into S phase.

Materials and Methods

Animals. (C57BL/6J × DBA/2J)F_1 (BDF_1) female mice were obtained from The Jackson Laboratory, Bar Harbor, ME. (CBA/N × DBA/2N)F_1 (CDF_1) male mice and (DBA/2N × CBA/N)F_1 (DCF_1) male mice were obtained from the Division of Research Services, National Institutes of Health, Bethesda, MD. Mice were used between 2 and 4 mo of age.

Reagents. Affinity-purified goat anti-μ antibody was prepared essentially as previously described (6). The serum of goat 759 was the source of the anti-μ. LPS w (*Escherichia coli* 0111:B4) prepared by the Westphal technique was obtained from Difco Laboratories, Detroit, MI. Fluorescent reagents used for analysis of cell surface markers (monoclonal anti-μ antibodies Bet 1 and Bet 2 [15], monoclonal anti-δ, 10.4.22 [16], monoclonal $14G8^2$, and a fluoresceinated mouse anti-rat IgG) were kindly provided by Dr. John Kung, National Institutes of Health.

Cell Preparation. Spleen cells were teased out of the spleen into Hanks' balanced salt solution (HBSS), washed, resuspended in HBSS containing a monoclonal anti-Thy-1.2 antibody (New England Nuclear, Boston, MA) and complement (guinea pig serum; 1:4, Flow Laboratories, Inc., Rockville, MD), and incubated at 37°C for 45 min to lyse the T cells. This treatment reduced the response of the cells to Con A to small percentage of that of untreated cells. Complement was removed by centrifugation and washing. The cells were then layered on top of a step gradient composed of 70, 60, and 50% Percoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc. Piscataway, NJ), respectively. In typical experiments 2.5 ml of each solution was layered in a sterile capped culture tube (2057; Falcon Labware, Div. of Becton, Dickinson, and Co., Oxnard, CA). Percoll was made isoosmotic by the addition of 10× phosphate-buffered saline (PBS); this solution was considered to be "100% Percoll," as defined by Pharmacia. This solution was diluted to 70%, the pH was adjusted to 7.4 with HCl, and the osmotic strength, as measured by freezing point depression with an Osmette S (Precision Systems, Inc., Sudbury, MA), was adjusted to be between 0.310 and 0.320 osmolal. The 50 and 60% Percoll solutions were obtained by diluting the 70% Percoll solution with HBSS (without $NaHCO_3$, adjusted to pH 7.2). Similar results were obtained when the Percoll was diluted with PBS, a condition which is closer to being isoosmotic for mouse cells (17). The gradient was chilled on ice for at least 15 min and then centrifuged at 2,300 *g* for 12 min at 5°C. In a Falcon 2057 tube, up to ~10^8 cells could be applied to the gradient without a decrease in the purity of the separated cells. The cells at each interface were collected from above with a pasteur pipette. Each fraction

was diluted at least twofold with HBSS, pelleted by centrifugation, and then washed twice with HBSS and once with culture medium.

Cell Culture. Cells were cultured in the serum-free medium recently described by D. E. Mosier (Iscove's/F-12) (13). This medium is 50% Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), 50% Ham's F-12 medium (Gibco Laboratories) with the following additives: insulin (5 μg/ml; bovine crystalline zinc insulin; Gibco Laboratories), human transferrin (5 μg/ml; Sigma Chemical Co., St. Louis, MO), progesterone (3×10^{-8} M; Upjohn Laboratories, Kalamazoo, MI), 2-mercaptoethanol (5×10^{-5} M), gentamycin (50 μg/ml), and trace elements, $MnCl_2 \cdot 4H_2O$ (10 ng/liter), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (600 ng/liter), $NiSO_4 \cdot 6H_2O$ (70 ng/liter), H_2SeO_3 (1.9 μg/liter), $NaSiO_3 \cdot 9H_2O$ (71 μg/liter), $SnCl_2$ (50 ng/liter), $Na_3VO_4 \cdot 4H_2O$ (600 ng/liter), and $CdSO_4$ (10.4 μg/liter). For most studies 3×10^6 cells were cultured in 1 ml of medium in 16-mm Diam flat-bottomed tissue culture wells (3524; Costar, Data Packaging, Cambridge, MA). Similar results were obtained at cell densities ranging from 1×10^6 to 5×10^6 cells/well. Cultured cells were stimulated with goat anti-μ antibody (50 μg/ml), or with LPS (50 μg/ml). Cells were harvested by gentle pipeting with a Pasteur pipette and all of the cells in the well were analyzed directly. For determining the uptake of [^{3}H]thymidine, cells were cultured at 2×10^5 cells/well in flat-bottomed microtiter plates (3596; Costar). All cultures were incubated at 37°C in an atmosphere containing 5% CO_2. When it was desirable to prevent cells from completing the cell cycle and going from M phase back to G_1 phase, 25 ng/ml colcemid was added to the cultures 24 h after anti-μ. This dose of colcemid was found not to increase cell death significantly, whereas higher concentrations did.

Electronic Volume Analysis. The size distribution of B lymphocytes was determined by electronic analysis using a Coulter Counter (model ZB1) and a Coulter Channelyzer L (model C-1000; Coulter Electronics, Hialeah, FL). The cells were suspended in PBS and analyzed with the following settings: amplification, 1/2, aperture current, 2; 100-mm aperture; lower threshold, 10; upper theshold, 100. Cell volume was determined by using microspheres from Coulter Electronics (Hialeah, FL) for calibration. Generally, 40,000 cells were analyzed per sample and plotted with full scale equal to 4,000.

Flow Cytometric Analysis. The DNA content of individual cells was measured in a Coulter TPS-1 Cell Sorter. Briefly, cells were centrifuged and stained with 50 μg/ml propidium iodide (Calbiochem-Behring Corp., American Hoechst Corp., LaJolla, CA) in 0.1% sodium citrate and 0.1% Nonidet P-40 (NP-40) detergent (18). The hypotonic solution caused cell lysis, but left the nuclei intact, with propidium bound to DNA. Between 50,000 and 100,000 nuclei were analyzed for each sample. The amount of fluorescence emitted was proportional to the DNA content of individual nuclei. Cell cycle analysis was performed by computer assisted data analysis on the National Institutes of Health PDP-10 (Digital Equipment Corp., Marlboro, MA) by a planimetric integration method as previously described (19). This staining method has been criticized by Traganos et al. (20) because of a potential loss of cells in M phase. We have found that not to be a problem with the lymphocytes used in these experiments based on the same two criteria used by Traganos et al. (20). That is, colcemid did cause a significant increase in the fraction of cells in G_2 + M that does not occur if the nuclei from cells blocked in M phase are especially fragile, and microscopic examination revealed that the nuclei from these cells do stay intact despite the lack of an obvious nuclear membrane. Nuclei with metaphase chromosomes were observed in equal proportions before hypotonic lysis and after flow cytofluorimetry, indicating that the analysis did not result in the disruption of M phase nuclei.

Flow microfluorimetric analysis of cell surface fluorescence was performed by W. Leiserson (National Institutes of Health) using an FACS II (B-D FACS Systems, Mountain View, CA). Determination of the amounts of mIgM and of the antigen recognized by the monoclonal antibody 14G8 on individual cells was done by using rat monoclonal antibodies and a fluorescent-labeled mouse anti-rat IgG. In calculating the fraction of positively stained cells, the number of cells stained by the fluoresceinated anti-rat IgG alone was subtracted from the number of cells stained by the monoclonal antibody followed by the fluoresceinated anti-rat IgG. The median fluorescence intensity was determined by calculating the median fluorescence for cells with more fluorescence than the negative control. This number was then normalized

to gain 16. The amount of IgD expressed on the cells was determined by staining directly with fluoresceinated monoclonal anti-δ (10.4.22). No correction was made to discount cells binding antibody nonspecifically in determining the fraction of cells staining positively with this reagent. The median fluorescence intensity for mIgD is not comparable to the intensity for the other two antigens, because the staining conditions were quite different.

Results

Separation of B Lymphocytes by Density-Gradient Centrifugation. Splenic B lymphocytes are known to exist in several different states. One physical property in which they show variability is size. Melchers and his colleagues (12, 21) have recently emphasized that small, dense B lymphocytes have distinct activation requirements when compared with larger, blast-like cells and thus any study of B cell activation would be clearer if it employed cell populations initially separated on the basis of size and density.

Fig. 1 presents a size analysis of splenic B lymphocytes. Such cells consisted of a major population corresponding in size to small, dense lymphocytes and found in a single peak. A substantial number of larger cells were also present. These did not clearly resolve into a separate peak.

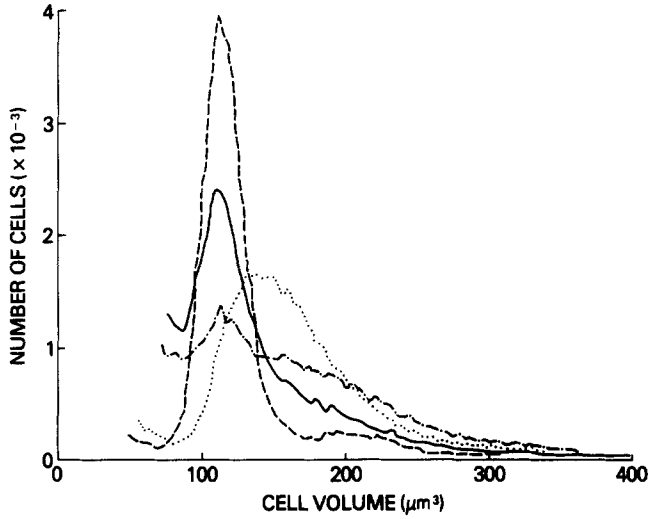

FIG. 1. Cell volume distribution of density-fractionated B cells. Spleen cells from BDF_1 mice were treated with anti-Thy-1.2 and complement and then separated on a Percoll step gradient. Fractions: low density (<1.062), above 50% Percoll; intermediate density (1.062–1.074), 50–60% Percoll; dense (1.074–1.086), 60–70% Percoll. Cell volume was determined by electric resistance and calibrated with microspheres obtained from Coulter Electronics.

TABLE I

Distribution of B Cells Recovered from Density-Gradient Centrifugation

Fraction	Density	Percent of recovered cells
Low density cells	1.062	16 ± 1%*
Intermediate density B cells	1.062–1.074	24 ± 2%
Small, dense B cells	1.074–1.086	60 ± 3%

The results are the average of 10 experiments with BDF_1 female mice.
* Mean ± SEM.

Cells were separated into populations of distinctive density by gradient sedimentation using Percoll as the density medium. Percoll has an inherently low osmotic strength and gradient separations using it are rapid, versatile, reproducible, and inexpensive. Between 50 and 65% of the starting cells were typically recovered from the gradient described here.

Cell populations separated by Percoll density-gradient separation had distinct size distributions as analyzed on a Coulter Counter. The dense fraction from Percoll separation (1.074–1.086 sp gr) was quite homogeneous in size (Fig. 1). Typically, 85–90% of these cells were in one sharp peak with a mean volume of 110 μm^3, conforming in size to the small cells found in the unseparated population. The cells of intermediate density (typically ~25% of the recovered cells) had a reproducibly larger size as measured electrically (mean volume, 140 μm^3). A similar difference between these two cell fractions was also observed by light-scatter analysis (data not shown). The cells of the lightest density fraction had a very heterogeneous size profile. The percent of cells recovered in each of these density ranges is shown in Table I.

The representations of mIgM, of mIgD, and of the membrane antigen recognized by 14G8 on the fractionated cells were determined by flow microfluorimetry. Results from representative experiments are presented in Table II. The cells applied to the Percoll gradient, having been treated with anti-Thy-1.2 antibody and complement, were predominantly B cells. The least dense fraction was somewhat depleted of B cells in comparison to the unfractionated cells. This is in agreement with the observations that this fraction was markedly enriched for phagocytic macrophages and for antigen-presenting cells (J. Mond, D. Longo, and L. Glimcher, unpublished observations). The B cells that were present in this fraction had somewhat more mIgM, as judged by median fluorescence intensity, than did the B cells of higher

TABLE II

Membrane Markers of Cells in Distinct Density Fractions

Cells*	mIgM‡		mIgD§		14G8 antigen‡		
	Percent of cells positive	Median fluorescence‖ intensity	Percent of cells positive	Median fluorescence intensity	Percent of cells positive	Percent of B cells¶ positive	Median fluorescence‖ intensity
Unfractionated	76	520	75	670	41	53	170
Low density	58	700	82**	490	43	74	430
Intermediate density	63	500	75**	420	45	71	250
High density	80	300	85	960	38 (23)‡‡	47 (29)‡‡	60 (150)‡‡

* Cells were obtained from $DCF_1♂$ mice. Similar results were obtained with cells from BDF_1 mice.

‡ Stained indirectly; the number of cells stained by mouse anti-rat IgG was subtracted from the number of cells stained indirectly to determine the percent positive cells.

§ Stained by direct means; no correction for nonspecific staining was made.

‖ Median fluorescence intensity of positive cells. The median fluorescence channel, normalized to the same gain (gain 16), is shown. These numbers are directly comparable for mIgM and 14G8 antigen, because the same second stain was used.

¶ Calculated by using the fraction of mIgM-positive cells stained in the same experiment as the number of B cells.

** The frequency of IgD-bearing B cells in the low density and intermediate density populations are probably substantially overestimated because of the binding of the directly fluoresceinated anti-IgD to the relatively frequent macrophages in these cell populations.

‡‡ Values in parentheses are the numbers obtained if the extremely dull staining cells (<channel 60 at gain 16) are not counted as positive.

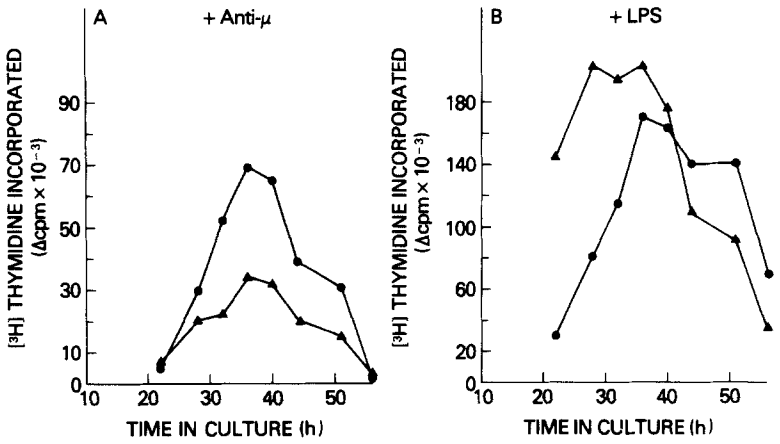

FIG. 2. Time-course of DNA synthesis by density-fractionated B cells stimulated with anti-μ (A) or LPS (B). B cells at 2 × 10^5 cells/well in Iscove's/F-12 medium were pulsed with [^{3}H]thymidine for 4 h at various times after the addition of anti-μ or LPS to dense B cells or to intermediate density B cells.

density. Indeed, cells in the high density B cell fraction had considerably less mIgM than did the unseparated cells or either of the less dense populations. On the other hand, these high density cells had approximately twice the amount of mIgD as did B cells of the other two fractions.

These fractionated B cells were even more distinctive with respect to their expession of the surface antigen recognized by the monoclonal antibody 14G8. Kung et al. have recently shown that this antigen is expressed only on a subset of B cells.[2] The two lighter density fractions contained B cells of which two-thirds to three-fourths were positive with 14G8. These cells displayed relatively intense fluorescence, with the least dense B cells being brighter than the intermediate density B cells. In contrast, only about one-half of the high density B cells were positive with 14G8, and many of the high density cells (~15%) expressed barely detectable amounts of the surface antigen. The median fluorescence of the brightest 25% of these cells was 150, still somewhat less than the median fluorescence for the intermediate density cells (250).

Response of B Cells Separated by Density to LPS and Anti-μ. B cell fractions separated by density were found to respond distinctively to anti-μ and to the B cell mitogen LPS. The high density B cells incorporated very small amounts of [^{3}H]thymidine in the first 24 h after addition of either activator (Fig. 2). In contrast, the intermediate density cells mounted a vigorous response to LPS at this time. In many experiments, a small but detectable response to anti-μ was observed at 24 h in cultures of intermediate density cells. Indeed, the response of the intermediate density cells to LPS was substantial as early as 12 h after addition of mitogen (data not shown). Furthermore, the intermediate density cells responded much more vigorously to LPS than to anti-μ, whereas in the dense B cell population the levels of response were more similar. In some experiments the anti-μ response of dense cells was greater than the LPS response, particularly when cells were cultured at 5 × 10^5 per well. The relatively late onset of DNA synthesis in the dense cell population suggests that it was mainly composed of B cells in the resting state. B cells of intermediate density, on the other

[2] Kung, J. T., S. O. Sharrow, A. Ahmed, R. Habberssett, and W. E. Paul, *J. Immunol.* In press.

hand, were induced to enter S phase much sooner than were the dense B cells, suggesting that these B cells were not in the resting state. These cells may represent cells that had become partially activated in vivo, or immature B cells that had not yet attained the resting stage of B cell differentiation, or a combination of both of these types of cells.

Size Analysis of Resting B Cells Stimulated with Anti-μ. The relatively homogeneous size of the dense B cells obtained by density-gradient centrifugation meant that the size changes accompanying the activation of these cells could be detected by volume analysis. Indeed, the Coulter Counter proved to be excellent for detecting size increases occurring in the course of B cell activation. B cells could be shown to be larger within 1 h after addition of anti-μ (Fig. 3). Furthermore, because each cell's volume was recorded as part of the histogram, it proved possible to analyze the frequency of resting B cells that enlarged in response to anti-μ. As can be seen in Fig. 3, virtually all small B cells increased their size upon stimulation with anti-μ. The enlargement of the stimulated cells appeared to occur at similar rates for all cells in the population. That is to say, the histograms of the stimulated and the nonstimulated cells both showed single peaks, although with different mean volumes. No evidence for two subpopulations enlarging at different rates could be found. Two distinctive populations would probably have resulted in a bimodal size distribution; this was not observed in any experiment among the >20 that were analyzed.

These size enlargements appear to represent activation of the cells from the resting G_0 state into and through the G_1 phase. This conclusion is supported by our preliminary results showing an increase in cellular RNA content of all dense B cells paralleling the size increase. Furthermore, the size enlargements correlate very well with the ability of these cells to enter S phase rapidly in response to a strong

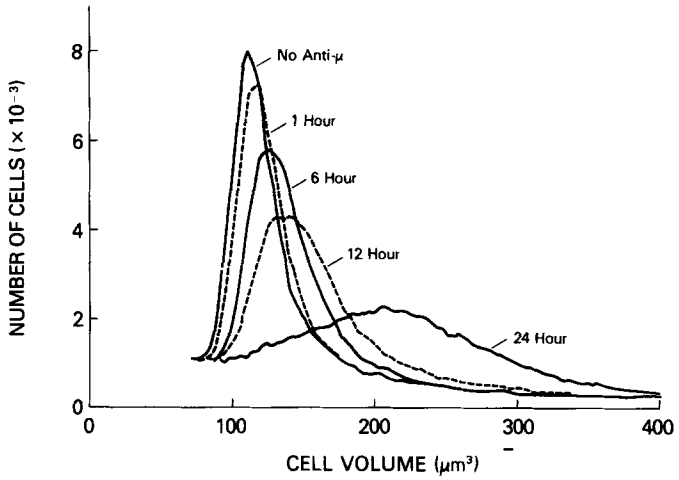

FIG. 3. Cell volume distribution of dense B cells stimulated with anti-μ. High density B cells prepared by density-gradient fractionation were cultured for various lengths of time in the presence of anti-μ and then subjected to cell volume analysis. Results of a typical experiment are shown. Cells cultured without anti-μ increased slightly in size soon after being put in Iscove's/F-12 medium, probably because the medium is hypotonic (0.290 osmolal). Subsequently, cell volume remained constant for at least 24 h. In this experiment anti-μ was added to the cells at different times before analysis, which was carried out on all samples at the same time. As a result, all cells were in culture for the same length of time.

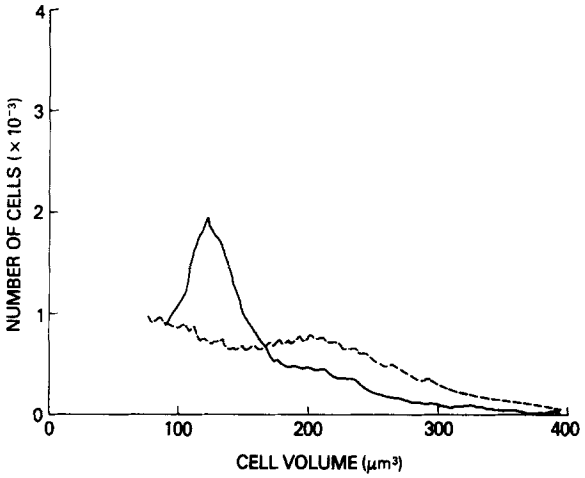

FIG. 4. Increase in size of high density $CDF_1♂$ B cells cultured with anti-μ for 24 h. Cells were cultured with or without anti-μ for 24 h in Iscove's/F-12 medium containing 0.15 mg/ml bovine serum albumin.

proliferative signal, such as that provided by LPS (A. L. DeFranco, E. S. Raveche, and W. E. Paul, manuscript in preparation).

These results strongly suggested that all resting B cells respond to anti-μ similarly in the first 24 h of stimulation. Previous results, however, had indicated that there are B cell populations that do not proliferate in response to anti-μ. One such population is the B cells from mice with the *xid*-determined B cell defect (10). These cells have been postulated to be similar to a subpopulation of normal B cells (22). Because of this, we examined the size changes of dense B lymphocytes prepared from CDF_1 male mice, which are hemizygous for the *xid* gene. These cells do not proliferate to a detectable degree in response to anti-μ. Nonetheless, many of these cells clearly enlarge upon exposure to anti-μ. Coulter analysis (Fig. 4) showed that many of the B lymphocytes from these mice enlarged in a manner comparable to that of B cells from phenotypically normal DCF_1 male mice.

Cell Cycle Analysis of B Cells Stimulated with Anti-μ. As described in the Introduction and in the previous section, certain B lymphocyte populations proliferate poorly or not at all to anti-μ. No direct measurements of the proportion of normal B lymphocytes that enter the cell cycle as a result of exposure to anti-μ have been reported. To address this issue it was necessary to analyze cells for entrance into S phase at the single-cell level. This was done by culturing high density B cells with anti-μ for varying times, lysing the cells at low ionic strength, staining intact nuclei with propidium iodide, a fluorescent dye that binds to DNA and gives a fluorescent signal proportional to DNA content, and analyzing them by flow microfluorimetry. Typical histograms of cell number vs. DNA content are shown in Fig. 5. These data were analyzed by computer to obtain the fraction of cells with a unit amount of DNA (cells in G_0 phase or G_1 phase), the fraction of cells with twice that amount of DNA (cells in G_2 phase or M phase), and the fraction of cells with intermediate amounts of DNA (cells in S phase).

Determination of the fraction of cells in S phase or in G_2 + M phases at various

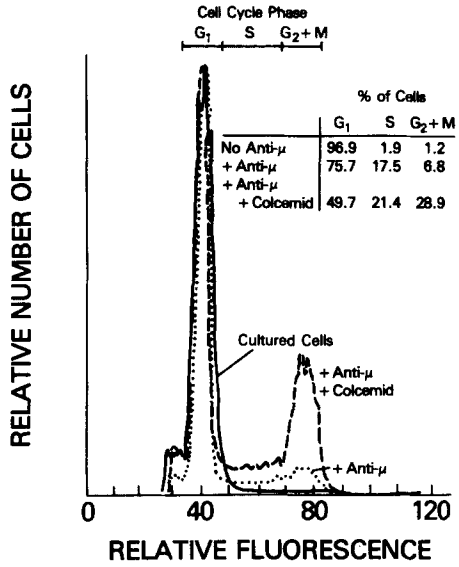

FIG. 5. Cell cycle analysis of high density B cells stimulated with anti-μ. Propidium iodide-stained nuclei from cells cultured for 48 h with or without anti-μ and with or without colcemid were analyzed by flow microfluorimetry. Colcemid (25 ng/ml), which blocks cells in mitosis, greatly increased the number of cells with twice the normal content of DNA.

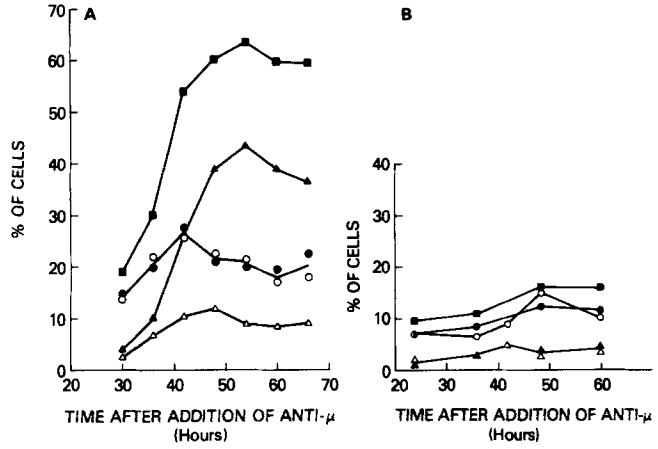

FIG. 6. The fraction of high density B cells entering S phase in response to anti-μ. Cells were cultured with or without colcemid, which prevented dividing cells from leaving M phase. Analysis for DNA content, as described in Materials and Methods, was carried out at different times after addition of anti-μ. These data were processed to determine the fraction of cells in different phases of the cell cycle. Cells with the normal amount of DNA are in G_0 or G_1 phases, cells with twice this amount of DNA are in G_2 or M phases, and cells with intermediate amounts of DNA are in S phase. The fraction of cells that have responded at any given time is equal to the sum of the cells in S, G_2, and M phases in the presence of colcemid, which prevented cells from returning to G_1 phase. Values for S phase and G_2 + M phases for cells cultured without colcemid were corrected for the increase in cell numbers due to cell division. (A) BDF_1 female, dense B cells. (B) CDF_1 male, dense B cells.

TABLE III

Fraction of Cells Entering S Phase in Response to Anti-μ and to LPS

Source	B cells fraction	Percent of cells responding		
		No mitogen	With anti-μ	With LPS
BDF_1	High density	8.1 ± 1.1 (9)*	56.4 ± 4.3 (7)	28.3 ± 9.9 (3)
BDF_1	Intermediate density	12.0 ± 2.8 (5)	40.5 ± 3.7 (4)	33.0 ± 4.1 (4)
DCF_1	High density	6.0 (3)	35.5 ± 1.8 (3)	13.7 ± 1.2 (3)
DCF_1	Intermediate density	9 (1)	30 (1)	27 (1)
CDF_1	High density	11.0 ± 1.9 (4)	12.0 ± 1.6 (4)	19.5 ± 1.5 (2)
CDF_1	Intermediate density	10.1 ± 2.0 (2)	11.0 ± 1.0 (2)	26 (1)

* Mean ± SEM. Number of experiments in parentheses.

times after addition of anti-μ made it clear that the B cells activated to transit through the cell cycle did so in a relatively unsynchronized fashion (Fig. 6 A). An analysis of the fraction of cells entering S phase from such data would necessarily underestimate the true fraction because some cells would have already gone through S, G_2, and M phases and returned to G_1 phase before other cells had entered S phase for the first time. To solve this problem, an inhibitor of mitosis (colcemid; 25 ng/ml) was added to the cells after 24 h of stimulation, before any had reached M phase. This prevented cells from completing the cell cycle and returning to G_1 phase. Thus, the fraction of cells entering S phase could be reliably determined simply by measuring the fraction of cells with greater than the unit amount of DNA (i.e., the number of cells in S plus the number in G_2 + M).

The response to anti-μ of dense B cells from BDF_1 mice is shown in Fig. 6 A. Typically, no cells entered S phase within the first 24 h after the addition of anti-μ. By 30–36 h, there was a large number of cells in S phase, and by ~40 h, these cells were accumulating in M phase, since transit through that phase was blocked by colcemid. By ~48 h in culture, all of the cells that would enter S phase had already done so. The fraction of cells in S phase was the same with or without colcemid. This demonstrates that colcemid did not perturb the cells before the finish of S phase. Thus, colcemid appears to be acting by blocking mitosis, as expected.

In contrast, B cells from the spleen of CDF_1 male mice (mice carrying an X chromosome with the defective *xid* allele) responded very poorly to anti-μ (Fig. 6 B), in agreement with previous results obtained by uptake of [^{3}H]thymidine (10). Typically, the population of CDF_1 male cells incubated with anti-μ contained only 1–2% more cells entering S phase than did the cells cultured without anti-μ (Table III). This increase was not statistically significant.

The average fraction of cells that entered S phase in response to anti-μ and to LPS in a series of experiments is shown in Table III. A large fraction of the cells prepared from BDF_1 mice responded to anti-μ. For the high density cell population, this represented ~48% of the cultured cells. Because B cells comprised ~80–85% of the high density cell population, nearly 60% of the B cells present were stimulated to enter S phase. By contrast, only 20% of the cells (25% of the B cells) in the high density population entered S phase in response to LPS. The intermediate density fraction also responded quite well to anti-μ; ~28% of all cells responded, or ~45% of the B cells. Approximately one-third of these B cells responded to LPS. The fraction of $DCF_1♂$ B cells responsive to anti-μ and to LPS was lower than the fraction of

responsive BDF_1 cells, although it was still a substantial proportion. Cells from $CDF_1♂$ animals did not show statistically significant responses to anti-μ but responded to LPS to the same extent as did the $DCF_1♂$ cells.

Discussion

We have examined the frequency of resting B lymphocytes that respond to anti-μ antibody in serum-free medium. B cells were fractionated on the basis of density to obtain populations that were more homogeneous than those normally obtained from the spleen. It was found that cells with densities between 1.086 and 1.074 (70% and 60% Percoll, respectively) are relatively uniform in size. Furthermore, these cells behaved like resting or G_0 state cells in that a long period of preparation, >24 h, was required before they entered S phase upon stimulation with LPS or anti-μ. Based on the recovery of cells from the density gradient, these cells accounted for 60% of spleen cells previously treated with anti-Thy-1 and complement. In addition to these resting B cells, B cells of intermediate density were also recovered. This intermediate density population contained nonresting cells, as some of them entered S phase within 12 h after the addition of LPS. These two fractions were also seen to be phenotypically different when the cell surface expression of mIgM, mIgD, and the antigen recognized by 14G8 were measured. The separation of these two physiologically distinct populations of B cells made it possible to study the activation properties of the cells, especially the resting cells, in more detail.

Virtually all high density B cells were observed to enlarge after being incubated with anti-μ. This enlargement was continuous and all B cells seemed to enlarge at roughly the same rate. There was no indication that there might be two different subpopulations with different rates of size increase. This enlargement probably reflects cell cycle advancement from the resting G_0 state to G_1 phase of the active cell cycle and preparation for entering S phase.

Although apparently all of the dense B cells enlarged upon stimulation with anti-μ, only ~60% of these cells from BDF_1 spleens and ~38% of these cells from DCF_1 spleens actually continued into S phase. Since 80–85% of the cells in these cultures were B cells, as defined by the presence of membrane IgM or IgD, these results suggest that ~25% of the BDF_1 cells and 45% of the DCF_1 cells were B cells that did not enter S phase in response to anti-μ. These calculations assume that cell death in culture was evenly distributed between responding and nonresponding cells. While probably an oversimplification, this assumption is supported by several lines of reasoning. The survival of cells for 2 d in Iscove's/F-12 medium was not markedly enhanced by the presence of anti-μ. Thus, responding cells did not appear to be exempt from cell death in culture. Furthermore, the cell size distributions indicate that all B cells, both those that will enter S phase and those that will not, enlarged at approximately equal rates, at least for the first 24 h. Any possible differences in viability would presumably not have occurred until after that time, when nonresponding cells became blocked in their transit through the cell cycle. Finally, the fraction of cells with greater than the diploid content of DNA did not increase between 48 h of culture and 60 h of culture. If the nonresponding cells were dying more rapidly, this fraction would have continued to increase. Nevertheless, the assumption we have made is presumably not entirely accurate, so the true fraction of cells that enter S phase is possibly somewhat biased in one direction or the other. Even if the responding or the nonresponding cells

account for all of the cell death, which is the most extreme possibility, the main conclusion of this work would not be changed: that is, that all B cells are equally stimulated to enlarge, whereas some cells are stimulated to enter S phase and some cells are not. For example, cell death in the first 48 h was generally just over 50%. If only cells that would not enter S phase died, then the starting population would represent ~25% responding cells and 75% nonresponding cells. Likewise, if only cells that would respond died in culture, then the starting population would represent 75% responding cells and 25% nonresponding cells. In either case, there would still be a sizable fraction of cells entering S phase and a sizable fraction of cells not entering S phase.

One interpretation of these results is that there are two functionally distinct subsets of B cells, one that can enter S phase in response to anti-μ and one that cannot. The second B cell subset is clearly affected by the presence of anti-μ, since these cells enlarge during the first 24 h and thus may be considered to have entered G_1 phase from the resting or G_0 state. These two subpopulations of B cells were not separated from one another by density fractionation, although small, resting B cells responded to anti-μ in somewhat greater numbers than did intermediate density B cells.

A major fraction of B cells, probably ~60% in BDF_1 spleen populations, do respond to anti-μ by proliferation. This is considerably higher than the proportion of B cells that respond to LPS. Previous estimates placed that number at 10–33% of the B cells, depending on the strain of mice used (23). Those results were obtained by limiting-dilution analysis in which cultures were scored as positive on the basis of the presence of antibody-secreting cells. In the experiments reported here, the fraction of cells responding to LPS by proliferation was measured and found to be similar to the previously reported fractions responding by antibody secretion. A similar result was also obtained by radioautography of cells incubated with [^{3}H]thymidine (24). It is striking that the percent of cells that enter S phase is not directly related to the amount of [^{3}H]thymidine incorporated. Thus, LPS causes much greater uptake of [^{3}H]thymidine by intermediate density cells than does anti-μ, even though anti-μ causes more of these cells to enter S phase. This difference may be explained by the rate at which cells responding to anti-μ and to LPS progress through S phase and/or by the frequency with which cells enter S phase for a second time.

Despite the finding that only a fraction of the high density B cells entered S phase in response to anti-μ, virtually all these cells undergo size enlargement. This suggests that a decision point exists in G_1 phase and that only some B cells can be stimulated by anti-μ to "choose" to go on to S phase. B cells from mice with the *xid* immune defect appear to exemplify those that enlarge but do not enter S phase in response to anti-μ. We should emphasize that those B cells from normal mice that enlarge but do not enter S phase in response to anti-μ are nonetheless capable of synthesizing DNA when appropriately stimulated because culturing dense BDF_1 B cells with anti-μ and LPS causes virtually all these cells to enter S phase (A. L. DeFranco, E. S. Raveche, and W. E. Paul, manuscript in preparation).

It seems likely that some mediator(s) from T cells or macrophages, or perhaps the direct action of histocompatibility-restricted T cells may act similarly to LPS in inducing proliferation in those B cells that enlarge but do not enter S phase in response to anti-μ. Indeed, it seems plausible that these other stimulants may act at the later G_1 decision point reached by cells stimulated by anti-μ. It is tempting to

suggest that antigen, like anti-μ, stimulates entry into G_1 by specific cells and either causes them to enter S phase or renders them sensitive to appropriate mediators or direct T cells signals, which in turn control entry into S phase.

Summary

The frequency of murine B lymphocytes that respond to antibodies directed against membrane IgM was measured. These anti-μ antibodies induced all, or almost all, resting B cells to enlarge over the first 24 h of stimulation. This probably represents the transition from the resting state (G_0) to active transit through the cell cycle. In contrast, only a fraction of these cells, ~60% for BDF_1 mice, continued through the cell cycle into S phase. This is consistent with previous experiments that had suggested there were some types of B cells that did not proliferate in response to anti-μ. The results presented here demonstrate that many, perhaps all, of these nonresponding B cells, both from normal mice and from mice with the *xid* defect, actually do respond to the presence of anti-μ by going through early parts of the cell cycle. These cells appear to become blocked at some point before the beginning of S phase, perhaps requiring a signal from a T cell or a macrophage to continue through the cell cycle. Thus, the role of antigen may be to prepare all B cells for proliferation. Different subpopulations of B cells may then require different regulatory signals before actually proliferating or before differentiating into antibody-secreting cells.

We thank Nibedita Mohanty for technical assistance, William Leiserson for help with the cell surface analysis, and Dr. John T. Kung for many helpful discussions.

Received for publication 8 September 1981 and in revised form 5 January 1982.

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. Adenolfi, M., B. Gardner, F. Gianelli, and M. McGuire. 1967. Studies on human lymphocytes stimulated *in vitro* with anti-γ and anti-μ antibodies. *Experientia (Basel).* **23**:271.
3. Gausset, P., G. Delespesse, C. Hubert, B. Bennes, and A. Govaerts. 1976. *In vitro* response of subpopulations of human lymphocytes. II. DNA synthesis induced by anti-immunoglobulin antibodies. *J. Immunol.* **116**:446.
4. Parker, D. C. 1975. Stimulation of mouse lymphocytes by insoluble anti-mouse immunoglobulins. *Nature (Lond.).* **258**:361.
5. Weiner, H. L., J. W. Moorehead, and H. Claman. 1976. Anti-immunoglobulin stimulation of murine lymphocytes. I. Age dependency of the proliferative response. *J. Immunol.* **116**:1656.
6. Sieckmann, D. G., R. Asofsky, D. E. Mosier, I. M. Zitron, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response. *J. Exp. Med.* **147**:814.
7. Sell, S., D. S. Rowe, and P. G. H. Gell. 1965. Studies on rabbit lymphocytes *in vitro*. II. Protein, RNA, and DNA synthesis by lymphocyte cultures after stimulation with phytohemagglutinin, with staphlococcal filtrate, with anti-allotype serum and with heterologous antiserum to rabbit whole serum. *J. Exp. Med.* **122**:823.
8. Kishimoto, T., and K. Ishizaka. 1975. Regulation of antibody response in vitro. IX. Induction of secondary anti-hapten IgG antibody response by anti-immunoglobulin and enhancing soluble factor. *J. Immunol.* **114**:585.

9. Parker, D. C., J. J. Fothergill, and D. C. Wadsworth. 1979. B lymphocyte activation by insoluble anti-immunoglobulin: induction of immunoglobulin secretion by a T cell-dependent soluble factor. *J. Immunol.* **123**:931.
10. Sieckmann, 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.
11. Sieckmann, D. G., R. Habbersett, I. Scher, and W. E. Paul. 1981. Activation of mouse lymphocytes by anti-immunoglobulin. III. Analysis of responding B lymphocytes by flow cytometry and cell sorting. *J. Immunol.* **127**:205.
12. 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.
13. Mosier, D. E. 1981. Primary *in vitro* antibody responses by purified murine B lymphocytes in serum-free defined medium. *J. Immunol.* **127**:1490.
14. Iscove, N. N., and F. Melchers. 1978. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *J. Exp. Med.* **147**:923.
15. Kung, J. T., S. O. Sharrow, D. Sieckmann, R. Lieberman, and W. E. Paul. 1981. A mouse IgM allotypic determinant (Igh-6.5) recognized by a monoclonal rat antibody. *J. Immunol.* **127**:873.
16. Oi, V. T., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr. Top. Microbiol. Immunol.* **81**:115.
17. Williams, N., and K. Shortman. 1972. The separation of different cell classes from lymphoid organs. The effect of pH on the buoyant density of lymphocytes and erythrocytes. *Aust. J. Exp. Biol. Med. Sci.* **50**:133.
18. Krishan, A. 1975. Rapid flow cytofluorimetric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell Biol.* **66**:188.
19. Gazdar, A. 1980. DNA content analysis by flow cytometry and cytogenetic analysis in mycosis fungoides and Sezary syndrome. *J. Clin. Invest.* **65**:1440.
20. Traganos, R., Z. Darzynkiewicz, T. Sharpless, and M. R. Melamed. 1977. Stimultaneous staining of ribonucleic and deoxyribonucleic acids in unfixed cells using acridine orange in a flow cytofluorimetric system. *J. Histochem. Cytochem.* **25**:46.
21. Melchers, F., J. Andersson, W. Lernhardt, and M. H. Schreier. 1980. H2-unrestricted polyclonal maturation without replication of small B cells induced by antigen-activated T cell help factors. *Eur. J. Immunol.* **10**:679.
22. Paul, W. E., I. Scher, J. J. Mond, A. A. Ahmed, B. Subbarao, and D. E. Mosier. 1978. B lymphocyte development, heterogeneity, and function. *Arthritis Rheum.* **21**:5175.
23. 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.
24. Janossy, G., M. F. Greaves, M. J. Doenhoff, and J. Snajdr. 1973. Lymphocyte activation. V. Quantitation of the proliferative responses to mitogens using defined T and B cell populations. *Clin. Exp. Immunol.* **14**:581.