FREQUENCIES OF MITOGEN-REACTIVE B CELLS IN THE MOUSE

II. Frequencies of B Cells Producing Antibodies Which Lyse Sheep or Horse Erythrocytes, and Trinitrophenylated or Nitroiodophenylated Sheep Erythrocytes

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The capacity of a B cell to grow and to mature into Ig-secreting, plaque-forming cells (PFC)[1] depends on its mitogen reactivity, i.e. to possess a receptor for a B-cell mitogen (1). Mitogen receptors must be distinct from Ig molecules on the surface of B cells, but they may be functionally and structurally associated with each other (2, 3). Every third B cell in the spleen of 6- to 8-wk old C3H/Tif mice has been found (4) to be reactive to the B-cell mitogen bacterial lipopolysaccharides (LPS) (5, 6) and approximately every fourth to the B-cell mitogen lipoprotein (7). Each mitogen-reactive B cell yields a clone of cells which secrete one set of variable (v) regions on Ig molecules and, therefore, Ig molecules with a given specificity for antigen. Therefore, mitogenic stimulation results in PFC secreting the entire repertoire of sets of v regions expressed in the pool of mitogen-reactive B cells. Since at least every third B cell in spleen is stimulated by LPS and lipoprotein this repertoire of stimulated clones of PFC may be a representative sample of the total repertoire of sets of v regions in all B cells.

The culture conditions which let every third murine splenic B cell develop in vitro into a clone of IgM-secreting PFC depend on the presence of 2-mercaptoethanol, a growth-supporting fetal calf serum, growth-supporting thymus cells, and a growth- and maturation-inducing B-cell mitogen, e.g. LPS or lipoprotein. The clones of Ig-secreting PFC can be assayed for their antigen specificities by a series of antigen-specific assays. In this paper, we have used a plaque assay for cells secreting hemolytic antibody to determine the number of mitogen-reactive B cells giving rise to clones of cells secreting antibody which, in the presence of complement, lyse sheep erythrocytes, horse erythrocytes, trinitrophenylated- or nitroiodophenylated sheep erythrocytes. In parallel, we have enumerated all clones of IgM-secreting cells in a modified hemolytic plaque assay which is independent of v-region specificities (7).

[1] *Abbreviations used in this paper:* B, bone marrow-derived, bursa equivalent; HRC, horse erythrocytes; Ig, immunoglobulin; LPS, bacterial lipopolysaccharides; NIP, nitroiodophenyl; PFC, plaque-forming cells; SpA, staphylococcus protein A; SRC, sheep erythrocytes; T, thymus-derived; TNP, trinitrophenyl; v, variable.

Materials and Methods

Animals. C3H/Tif/BOM-mice, 6- to 8-wk of age, were obtained from Gr. Bomholtgåard, Ry, Denmark. Lewis strain rats, 4 wk of age were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland.

Cells. Spleen cells, and growth-supporting thymus cells were prepared as described in the preceding publication (7). Rat thymus cells were used in all experiments as growth-supporting cells.

Culture Medium and Mitogens. Spleen cells in growth-supporting thymus cells (3×10^6 cell/ml) were grown in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N. Y.) containing 2-mercaptoethanol (5×10^{-5} M), fetal calf serum (10%, BioCult, Irvine, Scotland batch K 255701 D), and a B-cell mitogen in 5-ml plastic tubes in 0.2-ml aliquots (Falcon 2058; Falcon Plastics, Oxnard, Calif.) (see preceding publication). LPS-S (EDTEN 18735 and S435/188049) was kindly prepared for us by Doctors C. Galanos and O. Lüderitz, Max Planck Institut für Immunbiologie, Freiburg i. Br., West Germany. It was used at 50 μg/ml in culture. *Escherichia coli* lipoprotein (8), a gift of Dr. V. Braun, Mikrobiologie II, Universität Tübingen, Tübingen, West Germany, was used at 2 μg/ml.

Plaque Assays for Ig- and Antibody-Secreting Cells. For the detection of all cells secreting IgM a modified hemolytic plaque assay (9) was used which employs protein A (SpA)-coated sheep erythrocytes (SRC) and rabbit (anti-mouse IgM) antibodies as developing antibodies in the presence of properly diluted complement (BioCult). The IgM-specific antibodies were those described in the preceding publication. Protein A used in the coating of SRC was obtained from Dr. H. Wigzell, Biomedicum, University of Uppsala, Uppsala, Sweden.

Direct PFC with SRC, horse erythrocytes (HRC), trinitrophenyl (TNP)-SRC and nitroiodophenyl (NIP)-SRC were measured as described in previous publications (3, 6). TNP-groups were coupled to SRC by the method of Rittenberg and Pratt (10) with 30 mg or with 3 mg trinitrobenzene sulfonic acid per ml of packed, washed SRC. They are referred to as TNP_{30} SRC and TNP_3 SRC in the Results. NIP-groups were coupled to SRC by the method of Pasanen and Mäkelä (11) with 12 or 1 mg NIP-azide per ml packed, washed SRC. They are referred to as NIP_{12} SRC and NIP_1 SRC in the Results. NIP-azide was kindly prepared for us by Dr. H.-R. Kiefer from our Institute.

Results

In all antibody determinations, the definition of specificity is set by the detection assay. In these experiments we define as specific antibodies those which form hemolytic plaques in a Jerne-type assay.

All frequency determinations were done in vitro, at cell concentrations limiting the number of clones detected in the various plaque assays to around one per culture. According to Poisson's distribution, one B-cell precursor producing a clone with a given specificity is present in that number of cells per culture which let 63% of all cultures appear positive in the assay.

Spleen cells were diluted first in 3.3-fold dilutions, from 1×10^6 cells to one cell per culture, and grown in the presence of a constant number of growth-supporting rat thymus cells (3×10^6 per ml) and a growth-inducing mitogen, either LPS or lipoprotein. 10 cultures were assayed for each cell concentration on seven different target erythrocytes in the plaque assay, detecting clones secreting: (*a*) IgM, with protein-A-SRC and a rabbit anti-mouse IgM as developing serum, (*b*) SRC, (*c*) HRC, (*d*) TNP_{30}-SRC, (*e*) TNP_3-SRC, (*f*) NIP_{12}-SRC, and (*g*) NIP_1-SRC. Since the doubling time in clones of mitogen-reactive B cells is 18 h (4), each clone contains, at day 5 of culture, around 30–60 cells. Therefore, all cultures were assayed at day 5 and all those containing more than 15 PFCs were scored as positive, responding cultures. These screening experiments yielded the

TABLE I

*LPS- or Lipoprotein-Stimulated B-Cell PFC Responses Detected with Seven Different Plaque Assays at Different Concentrations of Spleen Cells Cultured in the Presence of Growth-Supporting Thymus Cells**

Number of spleen cells per culture	Numbers of PFC per culture‡ Mitogen: LPS							Mitogen: lipoprotein		
	SpA-SRC/anti IgM	$NIP_{12}SRC$	$TNP_{30}SRC$	NIP_1SRC	TNP_3SRC	HRC	SRC	SpA-SRC/anti IgM	$TNP_{30}SRC$	SRC
200,000	>2,000	>2,000	>2,000	>2,000	>2,000	>2,000	1,050	>2,000	>2,000	450
60,000	>2,000	>2,000	>2,000	>2,000	>1,000	600	210	>2,000	>2,000	80
20,000	>2,000	>2,000	>1,000	>1,000	750	185	65§	>2,000	>2,000	35§
6,000	>2,000	>2,000	550	370	180	55§	30§	>2,000	900	15§
2,000	>2,000	>1,000	185	180	80§	20§	<10	>2,000	210	<10
600	>1,000	520	65§	40§	25§	<10	<10	>1,000	65	<10
200	900	135	25§	<10	<10	<10	<10	600	15	<10
60	250	35§	<10	<10	<10	<10	<10	200	<10	<10
20	100§	<10	<10	<10	<10	<10	<10	75§	<10	<10
6	25§	<10	<10	<10	<10	<10	<10	20§	<10	<10

* 3×10^6 rat thymus cells/ml.

‡ Assay at day 5 of culture, average of 10 cultures.

§ Individual cultures fluctuating with either less than 10 PFC or more than 15 PFC, many ~60 PFC.

approximate number of cells per culture limiting the number of antibody producing clones to one, detected in the different plaque assays (Table I).

In the range of cell concentrations where antibody-producing clones were found to become fluctuating, indicating that the mitogen-reactive B cells producing antibodies with the tested specificities had become limiting, more narrow differences of cell concentrations were tested. Routinely, 40 cultures each were set up at 7–10 different cell concentrations within a range yielding between 10 and 90% nonresponsive cultures with the given plaque assay. At these limiting cell concentrations cultures were often found to contain around 60 PFC when assayed at day 5. Fig. 1 shows the distribution of clone sizes of IgM-secreting PFC which lyse SRC, determined in individual cultures containing numbers of reactive B cells limiting the SRC-specific clones to one, i.e. between 500 and 10,000 spleen cells per culture (see also Fig. 2). It is evident that the distribution is discontinuous and that a clone arising from one SRC-specific, LPS-reactive B cell contains approximately 60 PFC. Such clone sizes were expected from our previous results (1, 4), showing that B cells divide every 18 h and that every growing B cell within a clone at day 5 of culture secreted enough Ig to be detected in the plaque assay. The logarithm of the fraction of nonresponding cultures obtained at the different cell concentrations was then plotted against these cell concentrations in culture. Figs. 2A and 2B summarize the results of these experiments assayed with the seven different target erythrocytes in the plaque assay.

A linear degression of the logarithm of the fraction of nonresponding cultures

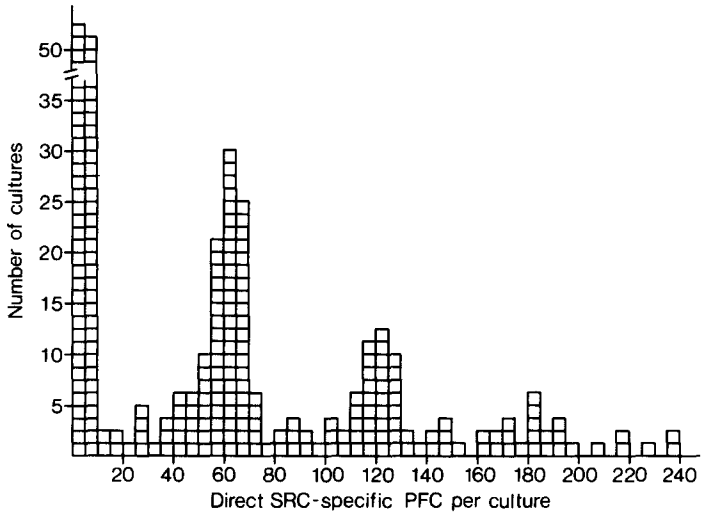


FIG. 1. Distribution of the number of direct SRC-specific IgM-secreting PFC in individual cultures of C3H/Tif splenic B cells grown in the presence of 50 μg/ml LPS and 3×10^6 rat thymus cells/ml. The total number of IgM-secreting, SRC-specific PFC was determined at day 5 of culture. One square represents one culture. Data are the cummulated values of all frequency determinations for SRC-specific B cells shown in Fig. 2 A.

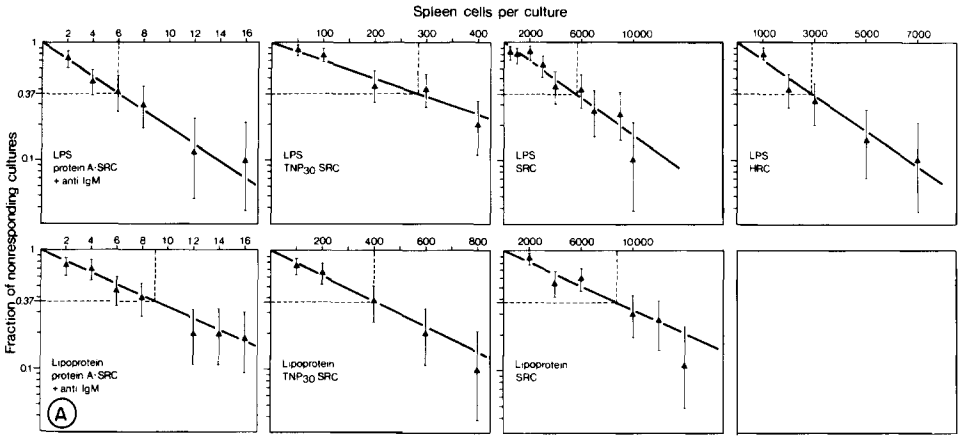
with increasing numbers of cells in cultures was observed in all cases for B-cell clones stimulated by either LPS or lipoprotein. This indicated that all B cells, stimulated by the two B-cell mitogens to clones of secreting cells, as detected in the seven different plaque assays, were limiting in the cultures. The number of cultured cells containing one B-cell precursor, developing into a clone producing antibody of the specificity set by the plaque assay was extrapolated from these data in Fig. 2 as that number of cells with which 37% of all cultures did not yield a positive response. These frequency determinations are summarized in Table II.

Since we can detect all IgM-secreting clones by the protein A plaque assay, we can calculate the absolute frequencies of specific B-cell clones in a given mitogen-reactive B-cell population. This is simply done by dividing the frequency of precursors giving a specific clone by the frequency of mitogen-reactive IgM-secreting cells (Table II).

We conclude from these data that the expected (1, 4) numbers of splenic B cells were stimulated to clones of IgM-secreting PFC by LPS (one of three B cells) or by lipoprotein (one of four to five B cells) (see preceding publication). LPS- and lipoprotein-reactive B cells appear to contain approximately the same number of B cells producing antibody detected in the SRC and the TNP_{30} SRC plaque assays. Higher coupling densities on SRC of TNP- and of NIP-haptenic groups yielded, as expected (2, 11), higher frequencies for B cells producing antibody detected in these plaque assays.

Discussion

The two B-cell mitogens, LPS and lipoprotein, each stimulate every third to fourth B cell in spleen of 6- to 8-wk old C3H/Tif mice, to clonal growth and



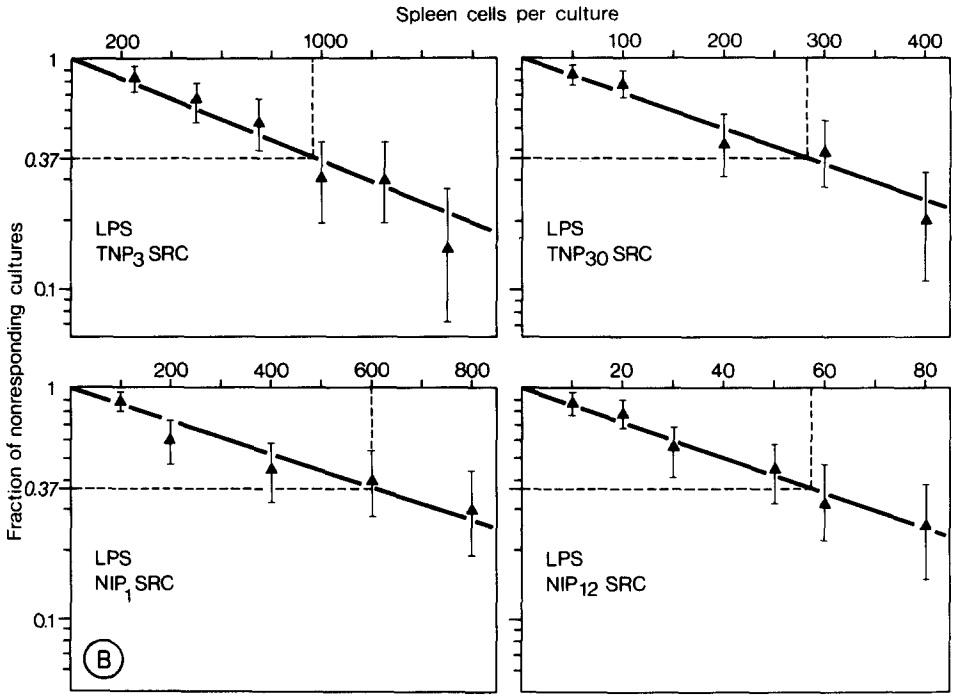


FIG. 2A. Titration of LPS- and of lipoprotein reactive B cells in C3H/Tif spleen. Cells were grown in the presence of 50 μg/ml LPS and 3×10^6 rat thymus cells/ml. Each point in the Figure is based on assays of 40 cultures. The assays were done (*a*) for total IgM-secreting PFC on protein A-SRC plus anti IgM-antibodies, (*b*) as well as for TNP_{30} SRC- (*c*) for SRC- and (*d*) for HRC-specific B-cell clones by the appropriate plaque assays on day 5 of culture. Vertical bars represent 95% confidence limits.

FIG. 2B. Titration of LPS-reactive B cells in C3H/Tif spleen, yielding clones of TNP- and NIP-specific PFC as assayed on SRC coupled with different densities of TNP- and NIP-determinants. Conditions for the experiments are those described in the legend of Fig. 2A. Vertical bars represent 95% confidence limits.

TABLE II
Frequencies of LPS- and of Lipoprotein-Reactive B Cells Producing Immunoglobulin Molecules Detected in Seven Different Plaque Assays

Plaque assay	Mitogen	Frequency* per total spleen cells	Approximate frequency* per B cells in spleen‡	Frequency* of specific B cells within the population of mitogen-reactive B cells§
Protein A-SRC and anti IgM-developing antibodies	LPS	1 in 6	~1 in 3	–
	Lipoprotein	1 in 8	~1 in 4	–
$NIP_{12}SRC$	LPS	1 in 57	~1 in 30	1 in 10
$TNP_{30}SRC$	LPS	1 in 280	~1 in 150	1 in 50
	Lipoprotein	1 in 400	~1 in 200	1 in 50
NIP_1SRC	LPS	1 in 600	~1 in 300	1 in 100
TNP_3SRC	LPS	1 in 960	~1 in 500	1 in 160
HRC	LPS	1 in 2,900	~1 in 1,500	1 in 500
SRC	LPS	1 in 5,700	~1 in 3,000	1 in 1,000
	Lipoprotein	1 in 9,400	~1 in 5,000	1 in 1,000

* Extrapolated from data in Fig. 1 for the cell concentrations at which 37% of all cultures are not responding.
‡ Assuming approximately 50% of all splenic lymphocytes to be B cells.
§ As detected by IgM-PFC in the protein A-SRC plaque assay.

secretion of IgM (7). This result has been verified with the experiments reported in this paper. At present, we do not know whether LPS- and lipoprotein-reactive cells are the same, i.e., whether both mitogen receptors are present on each B cell, or whether two distinct subpopulations carry only one of the two mitogen receptors each. Therefore, at least every third B cell in spleen was analyzed in our frequency determinations. This is the case if LPS and lipoprotein activate the same subpopulation of B cells. If LPS and lipoprotein-reactive B cells belong, however, to two different B-cell subpopulations, then we have analyzed the repertoire of two thirds of all B cells in the spleen. We found that the absolute frequencies of anti-SRC and anti-TNP_{30} SRC B cells were the same among LPS- and lipoprotein-reactive cells. If we activate two different subpopulations of B cells with LPS and lipoprotein, these results indicate that the v-gene repertoire is the same in two B-cell subsets.

It has been suggested that T-cell-independent and T-cell-dependent antigens stimulate different subpopulations of B cells to growth and maturation to Ig secretion (12–14). This would mean that receptors for T-cell help are on different B cells than the mitogen receptors for the mitogenic principles of T-cell independent antigens, including LPS and lipoprotein.

In this case our analyses of the repertoire of Ig molecules produced by different B cells would be restricted to those B-cell subpopulations which recognize the mitogenic principle of LPS and/or lipoprotein in T-cell independent antigens. If, however, the same, LPS- and/or lipoprotein-reactive B cells can also be stimulated by T-cell help, then our repertoire analyses would also cover those B cells which simultaneously express receptors for T-cell help.

How representative of the total repertoire of specificities are these frequency determinations? At present, there is no indication for a selective expression of certain v genes in different subpopulations of B cells, as defined by markers independent of Ig, in our case, the expression of a particular mitogen receptor. We, therefore, assume that our frequency determinations are representative of the total repertoire of Ig specificities. If not, it follows that none of the B-cell subsets, defined by functional reactivities to T-cell dependent or T-cell independent antigens will be representative of the total repertoire.

An experimental finding which suggests similarly high frequencies of specific B cells are the number of background PFC in normal mice. A fraction of all spleen cells are large, Ig-secreting lymphocytes. These cells are detected in the hemolytic plaque assay as background PFC. Whatever the origin of these background PFC is, idiotype interactions, B-cell stimulation by endogenous mitogens, or a necessary process of terminal differentiation for some B cells, they may be taken as representatives of the total repertoire of specificities, in the absence of overt antigenic priming. Table III shows the numbers of PFC in spleen of 6- to 8-wk old C3H/Tif mice, secreting IgM and secreting antibodies of the various specificities analyzed in our different plaque assays. The relative frequencies of specific antibody-producing cells to the total number of IgM producing cells are very similar to the absolute frequencies of mitogen-reactive B-cell precursors (compare Tables II and III).

The frequencies of B cells producing antigen-binding Ig of different specificities which we have determined for the LPS- and the lipoprotein-reactive populations of B cells can be compared to frequency determinations for B cells producing Ig binding to the same antigens detected with the same assays but under different experimental conditions for growth and maturation of B-cell clones. In vivo adoptive transfer of spleen cells often in the presence of antigen-primed T cells, into irradiated hosts, followed by challenge with antigen (15, 16) and in vitro Mishell-Dutton type (17) cultures of spleen cells in the presence of antigen-primed T cells in microcultures, again stimulated by antigen (13, 18) both yield 50- to 100-fold lower frequencies for the number of B cells producing antigen-binding Ig. These differences in frequencies cannot be due to different sensitivities in the assays, since in all experiments which we compare here, the same hemolytic plaque assays have been employed. In fact, for the detection of clones producing SRC-binding Ig we have used SRC from the very same sheep donor in practically the same plaque assay that was used in the frequency determinations of Quintans and Lefkovits (18–20) in microcultures.

We offer three explanations why our frequency determinations yield 50- to 100-fold higher numbers than do in vivo adoptive transfer experiments or in vitro Mishell-Dutton type microcultures. (*a*) We have determined frequencies under conditions where every growth inducible B cell has been demonstrated to

TABLE III
Specificity of Background IgM PFC in Normal Spleen

Plaque assay	Number of PFC per 10^6 spleen cells*	Ratio of specific PFC to total IgM-secreting PFC
Protein A-SRC	10,000	–
SRC	5	1 in 2,000
$NIP_{12}SRC$	300–600	1 in 16–32
$TNP_{30}SRC$	50–100	1 in 100–200

* 6- to 8-wk old C3H/Tif mice.

initiate growth (7). In all previous studies of lymphocyte responses in vitro, it is questionable whether all cells which actually had the capacity to be stimulated, did in fact initiate a response. This was largely due to the fact that growth could not be assessed by an increase in cell numbers, and B-cell maturation could not be measured by the total number of Ig-secreting cells. Therefore the frequency of stimulated clones could not be determined and thus the frequency of a defined specificity could not be corrected for plating efficiency. In our case, the absolute frequency of, e.g. anti-SRC precursor B cells −1/1000, is corrected from the experimental value −1/6,000, by comparing it with the frequency of all spleen cells initiating growth and IgM secretion i.e. 1/6 (Table II). As we have shown before, the fraction of all competent precursors which respond is largely dependent on the culture conditions (1). The anti-SRC PFC responses, originally reported to be in the range of 5,000 PFC per culture (17), have recently been improved, in the very same system, by factors exceeding 50-fold (21). This, to us, indicates that a different number of precursor cells initiate a response. Therefore, it seems likely that the 50- to 100-fold differences in frequencies of SRC specific B cells determined in Mishell-Dutton type systems (17) and in our conditions are due to our better culture conditions. (*b*) As discussed above, we can assume separate subpopulations of B cells reactive to LPS/lipoprotein and to T-cell help, all possessing the same v-region repertoire. If T-cell help-reactive B cells are 50–100 times less frequent than are LPS/lipoprotein-reactive cells, then the frequency of any B-cell specificity will be accordingly 50–100 times lower. (*c*) In vivo adoptive transfer experiments and in vitro microcultures are dependent on the presence of antigen to elicit a response. Our experiments, therefore, differ basically in the mode of activation of B cells, not in the assays for the produced Ig molecules. It may be that activating the cell with a mitogen, and therefore bypassing the affinity-dependent binding step of the antigen to the Ig receptors, we stimulate cells which secrete antibodies of high enough avidity to lyse the target erythrocytes, but still below the affinity threshold for activation. It follows that the antigen-dependent activation step, in some instances, selects a lower number of antigen-binding cells for growth and maturation, while mitogens, at their optimal concentration for B cells, always activate all cells. This explanation, however, is unlikely to account for a 50-fold difference in frequencies of specific B cells since the addition of LPS together with the antigen in the

Mishell-Dutton type of microculture experiments did not significantly change the frequencies of antigen-specific precursor B cells (19).

An increasing number of LPS-stimulated, growing B-cell clones can be detected by plaque assays when the epitope density of haptenic determinants, whether TNP- or NIP-, is increased on the SRC. Multivalent binding to the haptenated SRC of Ig molecules with low affinity for the haptenic determinants is likely to occur more frequently with increasing epitope densities. The frequencies of B cells producing Ig molecules binding to these haptenated SRC preparations agree well with numbers of PFC in mass cultures relative to the total number of PFC detected in the protein-A-SRC assay (1). The numbers of PFC in such mass cultures of mitogen-stimulated B cells are, therefore, reasonable first estimates for the number of reactive, Ig-producing B cells detected by the given assay. The high frequencies of B cells detected with densely coupled TNP_{30} SRC or NIP_{12} SRC justify their previous use to detect polyclonal B-cell maturation (2, 3, 6). It emphasizes how doubtful evaluations of antigen-specific responses must be which use these plaque assays (22–24). The definition of an antigen-specific response, thus, appears highly arbitrary. In antigen-stimulated responses specificity is probed in the system at least on two levels: first during the activation of B cells and later, in the final assay, at the level of the effector function. This must make quantitative evaluations of specificity as the contributions of affinities of interactions of sets of v regions with antigen to B-cell growth very difficult, if not impossible. Stimulating B cell by their growth-promoting mitogens appears better for evaluations of the repertoire of B cells since quantitations depend solely on the assays.

The surprisingly high frequencies of specific B cells appear to disagree with frequencies of serum antibody molecules with the same specificities within the total pool of natural serum Ig. If natural serum Ig constitutes a representative sample of the repertoire of all B cells, we would expect from our experiment, e.g., one in every 1,000 Ig molecules in normal mouse serum to lyse SRC. At present we have no indications that this is so. Since, however, we measure only IgM antibodies which constitute less than 10% of all natural serum Ig, it may well be that the same high frequencies could be found in the IgM fraction of natural antibodies. This raises the possibility that repertoires of IgM- and of IgG-producing B cells are different.

Summary

The B-cell mitogens LPS and lipoprotein stimulate 20–35% of all B cells in the spleen of 6- to 8-wk old C3H/Tif mice, as determined by limiting dilution analysis of precursors. Each reactive cell grows to a clone of IgM-secreting PFC, enumerated in a hemolytic plaque assay detecting all IgM secreting cells, regardless of v-region specificity. We have used these mitogens to reveal the total repertoire of Ig specificities produced by these mitogen-reactive B cells. We have determined in plaque assays with six different target erythrocytes the number of spleen cells limiting to one the number of mitogen-reactive B cells detected as specific IgM-secreting clones in each of these plaque assays. By this method, the absolute frequencies of precursor B cells with defined v-gene specificities could be calculated, for at least, one third of all B cells.

The frequencies of specific IgM-plaque-forming B-cell clones within the total pool of mitogen-reactive B cells was 1 in 10 for NIP_{12}-SRC, 1 in 50 for TNP_{30}-SRC, 1 in 100 for NIP_1-SRC, 1 in 160 for TNP_3-SRC, 1 in 500 for HRC, and 1 in 1,000 for SRC. These frequencies were the same in the LPS- and in the lipoprotein-reactive B-cell population for TNP_{30}- SRC and SRC.

We thank Miss H. Campbell and Mrs. C. Giacometti for able technical assistance.

Received for publication 28 February 1977.

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