ABSOLUTE FREQUENCIES OF LIPOPOLYSACCHARIDE-REACTIVE B CELLS PRODUCING A5A IDIOTYPE IN UNPRIMED, STREPTOCOCCAL A CARBOHYDRATE-PRIMED, ANTI-A5A IDIOTYPE-SENSITIZED AND ANTI-A5A IDIOTYPE-SUPPRESSED A/J MICE

By KLAUS EICHMANN, ANTONIO COUTINHO, and FRITZ MELCHERS

(From the Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, Heidelberg, West Germany and the Basel Institute for Immunology, Basel, Switzerland)

The B-cell mitogen lipopolysaccharide (LPS) (1) stimulates every third B cell in spleen of A/J-mice to clonal growth and IgM secretion in vitro (2, 3). Each clone secretes one set of variable (v)[1] regions on Ig molecules and, therefore, Ig molecules with a given specificity for antigen. All clones of IgM-secreting, plaque-forming cells (PFC), stimulated by LPS, secrete the entire repertoire of sets of v regions expressed in the pool of LPS-reactive B cells, i.e. in one third of all splenic B cells. The repertoire can be defined in quantitative terms as the frequency of B cells which are precursors for clones secreting IgM with a given specificity. These frequencies are determined in vitro by analyses limiting the number of specific precursors in culture to one, and can be correlated to the total number of LPS-reactive B cells. By this method we have previously determined within the pool of LPS-reactive B cells, the absolute frequencies of IgM-secreting, PFC clones-specific for sheep and horse erythrocytes, trinitrophenylated and nitroiodophenylated sheep erythrocytes (4).

In this paper we determined by the same method the approximate frequency of B-lymphocyte precursors for cells secreting IgM molecules which bear the A5A idiotype. This idiotype defines the major species of antibody molecules secreted by strain A/J mice in response to immunization with group A streptococcal vaccine (Strep A) (5, 6). The A5A idiotype is detected by guinea pig antisera against a monoclonal antibody to group A streptococcal carbohydrate (A-CHO) produced by irradiated A/J mice that have been reconstituted with small numbers ($\sim 10^6$) of pooled A/J spleen cells, and were subsequently immunized with Strep A (6).

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[1] *Abbreviations used in this paper:* A5A, strain A/J, idiotypically defined, anti-A-CHO antibody; A-CHO, group A streptococcal carbohydrate; B, bone marrow derived-, bursa equivalent-; IBC, idiotype-binding capacity; Id, idiotype; Ig, immunoglobulin; LPS, bacterial lipopolysaccharides; PFC, plaque-forming cell; SRC, sheep erythrocytes; Strep A, group A streptococcal vaccine; v, variable.

The A5A antibody is also defined by its spectrotype on isoelectric focusing (7) and the combined use of this method and of anti-idiotypic antisera revealed that over 90% of all A/J mice produce the A5A antibody after immunization with Strep A (5–7). Within the total antibody produced, the A5A idiotype accounts for about 25% (7, 8). Presensitization of A/J mice with the IgG1 fraction of anti-A5A antisera before the injection of Strep A leads to an increase in this proportion to close to 100% indicating that this class of anti-idiotype antibody can expand the precursor pool of A5A-positive B cells and, correspondingly, induces A-CHO-specific memory (8, 9).

In contrast, pretreatment of A/J mice with the IgG2 class of anti-A5A antisera leads to a drastic decrease of the proportion of A5A-positive antibodies upon subsequent immunization with Strep A (7, 10), and, by using low doses of IgG2 antibodies, this suppression becomes permanent and is maintained by suppressor T cells (10).

In this system, we have determined frequencies of precursor B cells-producing A5A idiotype in vitro to ask the following questions: (*a*) What is the frequency of A5A-bearing precursor B cells in normal mice? (*b*) Does the induction of A-CHO-specific memory result in an increase of the frequency of A5A-bearing precursor B cells and is the difference in the idiotypic heterogeneity of memory cells induced by IgG1 anti-A5A and by Strep A reflected in the respective precursor frequencies? (*c*) Is the state of idiotype suppression, which is actively maintained by suppressor T cells, reflected by a decreased frequency for precursor B cell? (*d*) How many of the B-cell clones that bear the A5A idiotype possess specificity for A-CHO? We can answer each of these questions in quantitative terms, by testing the supernates of LPS-stimulated spleen cell cultures at limiting cell concentrations for the presence or absence of A5A idiotype and analyzing the data according to Poisson's distribution.

Materials and Methods

Animals. A/J-mice were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland, or from Gr. Bomholtgaard, Ry, Denmark. Male or female mice, 4–10 wk old, were used in all experiments. Lewis strain rats were obtained from the Institut für Biologisch-Medizinische Forschung.

Anti-Idiotype Antisera. Anti-idiotype (Anti-Id) antisera to antibody A5A were prepared in guinea pigs that were rendered tolerant to mouse IgG as previously described (6, 11). IgG1 and IgG2 fractions from these antisera were obtained by agarose block electrophoresis as described (7).

Immunizations. Mice were immunized either with 1–2 × 10^9 Strep A organisms (5, 6), or with the IgG1 and IgG2 fractions, respectively, of an anti-A5A idiotype antiserum (8). IgG1 and IgG2 fractions were calibrated according to their binding capacity IBC and 0.1 μg IBC/mouse was injected, as previously described (8).

Cells. Spleen cells and growth-supporting thymus cells were prepared as described previously (2). Rat thymus cells were used in all experiments as growth-supporting cells.

Culture Medium and Mitogen. Spleen cells with growth-supporting thymus cells (3 × 10^6 cells/ml) were grown in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing glutamine (2 mM), penicillin, and streptomycin (5,000 IU/ml each), HEPES-buffer (10 mM), pH 7.3, 2-mercaptoethanol (5 × 10^{-5} M), fetal calf serum (10%; BioCult, Irvine, Scotland, batch K 255701 D), and LPS (50 μg/ml). LPS-S (EDTEN 18735 and S 435/188049) was kindly prepared for us by Doctors C. Galanos and O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, W. Germany. Cultures were kept either in Costar tissue culture trays (no. 3524, Costar, Data Packaging Corp., Cambridge, Mass.), in 1-ml aliquots, or else in Falcon Microtest II-plates (Falcon Plastics, Div. of Bioquest, Oxnard, Calif. no. 3040) or in 5-ml Falcon tubes (no.

2058), in 0.2-ml aliquots, in a humidified 37°C incubator gassed with a mixture of 5% CO_2 in air. The efficiency of growth and maturation to IgM-secreting cells was assayed in each experiment at day 5, in 10 separate cultures with spleen cell concentrations limiting the number of LPS-reactive B cells developing an IgM response to one (see Results). For the analysis of A5A idiotypes, supernates were collected by centrifugation after 10–14 days of culture. The supernatant medium could be kept frozen until it was analyzed for secreted A5A idiotype.

Plaque Assay for Secreting Cells. A modified hemolytic plaque assay with protein A-coated sheep erythrocytes (SRC) and mouse Ig-specific antisera as developing antibodies in the presence of complement was employed to detect and enumerate all Ig-secreting cells (12). IgM-secreting PFC were scored by employing specific antisera which were raised by repeated injections of 0.5–1 mg purified MOPC 104 E 19S IgM in Freund's incomplete adjuvants. The antisera detected μH- and λL-chains, but not γH or αH or κL-chains secreting cells as tested in plaque assays with corresponding myeloma tumor cells. Protein A used in coating of SRC was obtained from Dr. H. Wigzell, Biomedicum, University of Uppsala, Uppsala, Sweden. Complement, appropriately diluted with BSS, was from BioCult.

Assay for A5A Idiotype. Determination of A5A idiotype: the quantitative determination of A5A idiotype in culture supernates employed our previously described radioimmune inhibition assay (5) in slightly modified form. 2–3 ng of radioiodinated-purified A5A antibody (15,000–20,000 cpm) was incubated with guinea pig anti-A5A antiserum at a dilution that binds about 70% of the radiolabeled A5A, in a total vol of 80 μl. To this, 100 μl of culture supernate was added and after a 4-h incubation, 20 μl of diluted rabbit anti-guinea pig antiserum was added. After overnight incubation, the radioactivity in the precipitates was determined as described (5). A standard inhibition curve was constructed by using cold A5A as inhibitor in concentrations ranging from 2 to 6,000 ng/ml. 50% inhibition is achieved at about 200 ng/ml. The concentration of A5A idiotype in each culture supernate equals that which causes the same degree of inhibition in the standard curve. Initially, a number of supernates were tested in 3.3-fold dilution series and, invariably, inhibition curves parallel to the standard curve were observed. Thereafter, a single point was considered sufficient to estimate the concentration of A5A idiotype in a culture supernate.

A supernate was considered positive for A5A idiotype when 100 μl of it inhibited more than 20% of the binding of the radioactive A5A to anti-A5A. This point in the standard curve is equivalent to 27 ng A5A/ml, i.e. 2.7 ng A5A/100 μl undiluted supernate. Thus, the limit of sensitivity for this assay is about 5–6 ng/culture, in a 0.2-ml culture. As will be seen later, these borderline concentrations were hardly ever observed, since a single clone produces around 30 ng A5A.

Detection of Anti-Strep A-Antibody in Culture Supernates. To determine whether the A5A idiotype in culture supernates was associated with antibodies to A-CHO, 50 μl of supernate was added to 1×10^{10} packed Strep A particles and another 50 μl was added to an about equal amount of packed *Bordetella pertussis* for control. The organisms were resuspended in the supernates and the tubes were rotated at 4°C for 1 h. After centrifugation, the amounts of A5A idiotype in both aliquots were determined by the radioimmune inhibition assay (see above).

Results

Frequencies of A5A Idiotype Producing B cells. All frequency determinations were done in vitro at cell concentrations limiting the number of clones producing either A5A idiotype or IgM to around one per culture. According to Poisson's distribution, one B-cell precursor for a clone of cells secreting a given type of IgM molecule is present in that number of cells plated in individual cultures which let 63% of all cultures appear positive in the assay.

We first screened spleen cells of unprimed, Strep A-CHO-primed, and anti-idiotype-primed mice for the approximate frequencies of A5A idiotype-producing B cells and for the frequencies of total IgM-secreting PFC clones in 3.3-fold dilutions from 2×10^5 cells to two cells per culture. 10 cultures were assayed at each concentration with the two tests. Data in Table I show that all spleen cell

TABLE I

LPS-Stimulated B-Cell Responses at Different Concentrations of Spleen Cells from Unprimed, Strep A-Primed and Anti-A5A Idiotype-Primed A/J Mice Detected as A5A Idiotype-Secreting Cultures and as IgM-Secreting, PFC-Positive Cultures

Number of spleen cells per culture	Source of spleen cells					
	Unprimed		Strep A-primed		Anti-A5A IgG1-Primed	
Assay:	IgM PFC	A5A idiotype	IgM PFC	A5A idiotype	IgM PFC	A5A idiotype
2×10^5	ND	10	ND	10	ND	10
6×10^4	ND	10	ND	10	ND	10
2×10^4	ND	10	ND	10	ND	10
6×10^3	ND	8	ND	10	ND	10
2×10^3	ND	1	ND	7	ND	10
6×10^2	10	1	10	5	10	8
2×10^2	10	0	10	0	10	2
6×10^1	10	0	10	0	10	1
2×10^1	9	0	10	0	10	0
6×10^0	6	ND	7	ND	5	ND
2×10^0	2	ND	2	ND	1	ND

Number of positive cultures in a total of 10 cultures tested. ND, not done.

preparations, either from unprimed or primed mice had, approximately the same number of LPS-reactive B cells, i.e. one in six spleen cells, as assayed by the development of IgM-secreting PFC clones. This absolute number of LPS-reactive B cells was also determined in all other experiments reported below and was found, in all cases, to be close to one in six spleen cells.

The approximate number of A5A idiotype-producing B-cell clones secreting into the culture medium idiotype detectable by the specific radioimmunoassay, were 1 in 6×10^3 spleen cells in unprimed mice, 1 in 2×10^3 in Strep A-CHO-primed mice and 1 in 6×10^2 in anti-A5A IgG1-primed mice (Table I).

In the range of cell concentrations where A5A idiotype production became fluctuating, indicating that the LPS-reactive A5A idiotype-producing B-cell precursors had become limiting, narrower differences of cell concentrations and larger numbers of cultures were tested. Table II summarizes the results from three experiments. We have pooled the data from these individual experiments for a given type of spleen cell source for the frequency determinations shown in Fig. 1, since in all experiments the number of LPS-reactive B cells stimulated to IgM-secreting PFC clones was almost the same, i.e. close to one in six spleen cells.

For all four cell types analyzed, the number of cultured spleen cells containing one A5A-positive B-cell precursor, developing into a clone of IgM-secreting cells after LPS stimulation, were extrapolated from the data in Fig. 1 as that number of cells with which 37% of all cultures did not yield a positive response. These frequency determinations are summarized in Table III.

Since we can detect all IgM-secreting clones stimulated by LPS we can correlate the frequencies of A5A idiotype-producing B cells to the total number

TABLE II

Number of LPS-Stimulated Spleen Cell Cultures with A5A Idiotype-Positive Supernates at Varying Numbers of Spleen Cells from A/J Mice that were either Unprimed or Pretreated with Strep A, Anti-A5A IgG1, and Anti-A5A IgG2

Number of spleen cells per culture	Unprimed			Strep A-primed			Anti-A5A IgG1-primed			Anti-A5A IgG2-primed		
	No. tested	No. positive	Percent positive	No. tested	No. positive	Percent positive	No. tested	No. positive	Percent positive	No. tested	No. positive	Percent positive
2×10^4										20	20	100
1.2×10^4	86	83	97									
1×10^4	70	63	90									
8×10^3	20	13	65									
6×10^3	80	25	39	30	29	97				20	3	15
4×10^3	178	37	21	20	18	90						
2×10^3	244	33	14	80	53	67	122	115	94	20	1	5
1.6×10^3							140	123	87			
1.2×10^3				50	11	22						
1.0×10^3							50	39	78			
6×10^2							60	20	33	20	1	5
2×10							60	4	6			
6×10										20	0	0

TABLE III

Frequencies of LPS-Reactive B-Cells Producing A5A Idiotype in Unprimed, Primed, and Suppressed A/J Mice

Source of spleen cells	Frequency*		
	In total spleen cells	Approximate in splenic B cells‡	Absolute within the population of LPS-reactive B cells§
Unprimed	$1:15.3 \times 10^3$	$1:7.6 \times 10^3$	$1:2.5 \times 10^3$
Strep A-primed	$1:1.7 \times 10^3$	$1:0.85 \times 10^3$	$1:2.8 \times 10^2$
Anti-A5A-IgG1 primed	$1:0.8 \times 10^3$	$1:0.4 \times 10^3$	$1:1.3 \times 10^2$
Anti-A5A-IgG2 primed	$1:40 \times 10^3$	$1:20 \times 10^3$	$1:6.7 \times 10^3$

* Extrapolated from data in Fig. 1 for the cell concentration at which 37% of all cultures did not respond.

‡ Assuming approximately 50% of all splenic lymphocytes to be B cells.

§ Determined, in the same experiments, by frequency determinations of IgM-secreting PFC clones.

of LPS-reactive B cells, and, therefore, can calculate the absolute frequencies of A5A idiotype-producing precursors within the pool of LPS-reactive B cells (one in six spleen cells, see above and [2, 3]) (Table III).

Of the four cell types analyzed, only the data obtained with cells primed with Strep A and with cells primed with anti-A5A IgG1 could be fitted to a linear degression of the logarithm of the fraction of idiotype-negative cultures, indicating that the A5A idiotype-producing B-cells were limiting in these cultures (Table II, Fig. 1). Normal unprimed cells showed a sharp drop in their degression line when the cell number exceeded 6×10^3/culture, and a similar drop was also observed with A5A-suppressed cells. We have analyzed this situation in more detail and have found that at a concentration of 3×10^4 cells/ml (6×10^3/culture), the culture supernates contain between 100 μg and 1 mg/

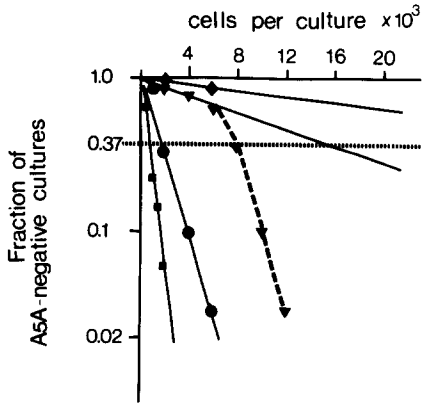

FIG. 1. Titration of LPS-reactive B cells of unprimed (▼), Strep A-primed (●), anti-A5A idiotype (IgG1)-sensitized (■) and anti-A5A idiotype (IgG2)-suppressed (◆) A/J mice. Cells were grown in the presence of 50 μg/ml LPS and 3 × 10^6 thymus cells/ml for 10–14 days and A5A idiotype-bearing Ig measured in the supernates of the cultures by a radioimmunoassay described in the Materials and Methods section. Each point in the Figure is based on the number of assays given in Table II. The dotted line (· · · · ·) gives the numbers of cells at which 37% of all cultures did not contain A5A idiotype, i.e. which, according to Poisson's distribution, yield that number of spleen cells in which one A5A idiotype-producing, LPS-reactive B precursor is present. The dashed line (-----) shows the actually measured curve for unprimed cells, and indicates where the assay quits to be linear, due to excess normal Ig interfering in the radioimmunoassay for A5A idiotype (see the text).

ml of non-A5A idiotype-bearing, nonspecific Ig. More than 100 μg of this Ig had to be added to the radioimmune inhibition assay (see below and Materials and Methods), to identify the small quantities of idiotype in these cultures. At this concentration of mouse Ig, the degree of precipitation between the rabbit anti-guinea pig Ig antiserum and the guinea pig anti-Id antiserum becomes incomplete (5), so that the test does no longer detect A5A idiotype only. This results in detection of false positives, consequently increasing the number of positive cultures to values higher than expected and in a rapid drop of the curves in Fig. 1. Nevertheless, the data obtained with unprimed and suppressed cells at cell concentrations lower than 6 × 10^3/culture (3 × 10^4/ml), fit a linear degression line and we have, therefore, extrapolated these degression lines in a linear fashion.

The Amount of A5A Idiotype Produced by Single Clones. The radioimmunoassay for A5A idiotype allowed a quantitative evaluation of the amounts of idiotype-bearing Ig secreted into the culture medium (see Materials and Methods). Fig. 2 shows the distribution of quantities of A5A idiotype produced in individual cultures which contained numbers of A5A idiotype-producing B-cell clones near one (see also Table II and Fig. 1). It is evident that the distributions for unprimed as well as for primed spleen cells are discontinuous. A5A idiotype-positive cultures accumulated around 25–30 ng, 55–60 ng, and 85–90 ng per culture. In most cases, the numbers of cultures under each of the observed peaks fit within the 95% confidence limits of those calculated with Poisson's distribution, with the assumption that the clonal progeny of a single A5A idiotype-bearing LPS-reactive B cell secreted 30 ng of A5A idiotype under

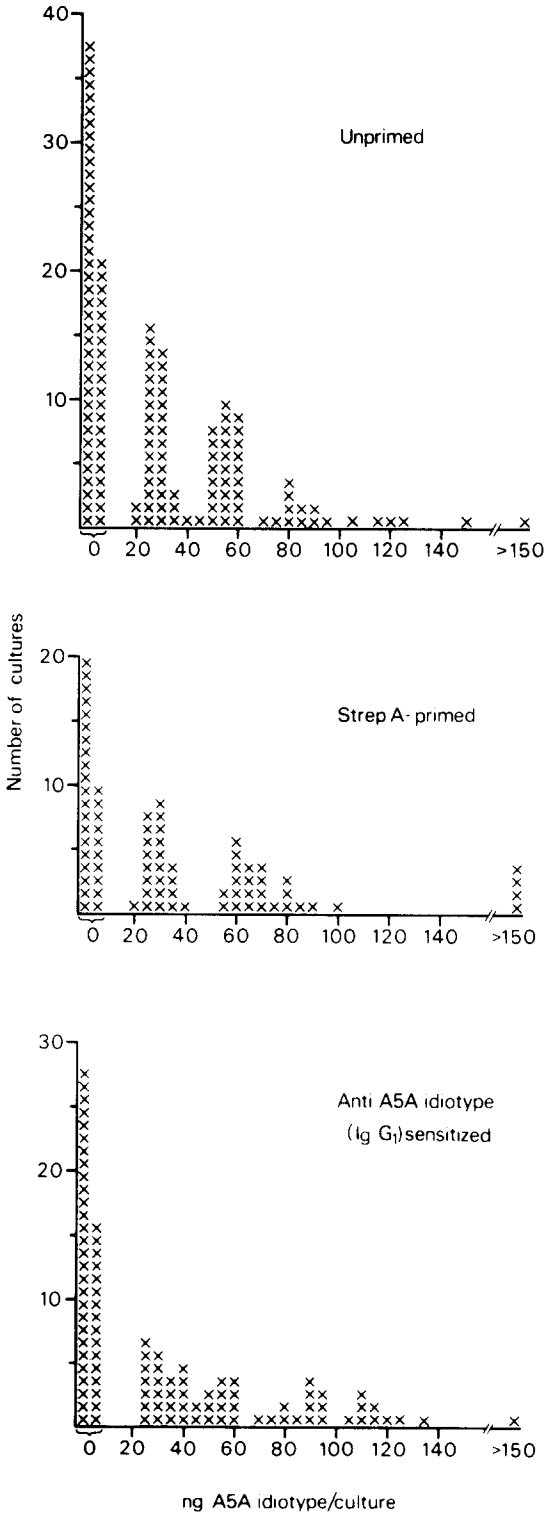
Unprimed
Strep A- primed
Anti A5A idiotype
(Ig G1) sensitized
Number of cultures
ng A5A idiotype/culture
0 20 40 60 80 100 120 140 >150

TABLE IV

Frequency of Cultures Developing None, One (30 ng), Two (60 ng), or Three (90 ng) Clones of A5A-Secreting, LPS-Reactive B Cells, at Cell Concentrations Limiting the Number of Reactive Cells to Around One Per Culture

Number of cultures with:	Cells in culture		
	Normal	Strep A-primed	Anti-A5A IgG1-primed
No clone	59:42% (32–50:40.6%)*	30:37.5% (28–46:36.7%)*	44:44% (35–54:44.9%)*
One clone	36:25% (27–45:36.5%)	23:28% (28–46:36.7%)	22:22% (27–44:35.9%)
Two clones	29:20% (9–24:16.5%)	16:20% (13–28:18%)	14:14% (9–22:14.4%)
Three clones	10:7% (2–10:5%)	7:9% (4–16:6%)	11:11% (1–9.5:3.8%)
>Three clones	6:4% (0–6:1%)	4:5% (2–10:2%)	10:10% (0–4.6:0.7%)
Total	140:100%	80:100%	100:100%

* Expected percent and 95% confidence limits of the experimental values in Poisson's distribution.

our culture conditions. These numbers and the 95% confidence limits are summarized in Table IV.

Association of A5A Idiotype with A-CHO Specificity. Since we can enumerate from the concentration of A5A idiotype in the supernate the number of A5A-secreting clones in each culture, we can analyze, at the single clone level, how many of the clones that secrete A5A idiotype possess specificity of A-CHO. This was done by absorbing equal volumes of supernates from single, double, and triple clone cultures to group A streptococcal organisms and, as a control, to *B. pertussis*. Thereafter, the supernates were analyzed for reduction in idiotype content.

The data on cultures from unprimed cells and from cells primed with Strep A and with anti-A5A IgG1 are summarized in Table V. The number of idiotype-positive clones whose antibodies absorbed to Strep A was between 40 and 50% of the total idiotype-positive clones analyzed for each cell type.

The quantitative absorption data obtained on single, double, and triple clone cultures supported the concept that each clone produced around 30 ng idiotype, as the absorption of idiotype clearly occurred in 30-ng portions and each

FIG. 2. Distribution of the amount of A5A idiotype-bearing Ig in individual cultures of unprimed, Strep A-primed, and anti-A5A-idiotype-(IgG1)-sensitized A/J spleen cells grown in the presence of 50 μg/ml LPS and 3×10^6 thymus cells/ml under culture conditions limiting the number of A5A-producing B-cell precursors to around 1 (see Fig. 1 and Table IV). A5A idiotype-bearing Ig was quantitated in the supernates of the cultures kept for 10–14 days by a radioimmunoassay described in the Materials and Methods section.

TABLE V

The Proportions of A5A Idiotype-Secreting Clones that Possess Specificity for A-CHO in Unprimed Cells and in Cells Primed with Strep A and with Anti-A5A IgG1

	Source of spleen cells		
	Unprimed	Primed with Strep A	Primed with anti-A5A IgG1
Number of A5A-bearing clones tested	131	167	129
Number of clones specifically absorbed to Strep A	58	77	50
Percent of A-CHO-specific among A5A-bearing clones	44.3	46.1	38.8

TABLE VI

*Absorption of Idiotype on Strep A Organisms in Supernates of Cultures that Contained One, Two, or Three Clones of A5A-Producing Cells**

Number of A5A-bearing clones/culture					
One clone		Two clones		Three clones	
A5A idiotype (ng/200 μl) after absorption on:					
B. pertussis	Strep A	B. pertussis	Strep A	B. pertussis	Strep A
40	0	52	28	80	60
32	0	60	28	88	60
28	0	56	28	92	60
28	0	64	32	88	56
36	0	64	36	88	64
32	0	60	32	80	32
32	0	56	0	96	28
36	0	56	0	92	36
28	0	60	0	92	28
32	0	52	0	92	0

* Only cultures which contained A5A idiotype absorbing to Strep A are shown on this Table.

portion absorbed independently of the others. 10 examples for absorptions of supernates containing A5A idiotype from one, two, or three clones are shown in Table VI.

Discussion

The B-cell mitogen LPS stimulates every third B cell of A/J-spleen to clonal growth and IgM secretion in vitro (2, 3). We have, therefore, analyzed with our frequency determinations the representation of A5A idiotype-producing cells in the LPS-reactive third of the total B-cell compartment. We cannot, at the present time, probe for the repertoires of the other two thirds of all B cells, since we have not yet identified the mitogens which stimulate them. It remains, therefore, the question as to how representative our frequency determinations are for the total pool of B cells. At the present, we have no indication that different B-cell subpopulations as defined by mitogen reactivity, selectively express different sets of v regions.

The frequency determinations of A5A idiotype-producing B cells in unprimed spleen add another number to our quantitative description of the repertoire of v gene specificities in normal murine B cells, previously done for SRC-, horse erythrocytes, trinitrophenylated-SRC- and nitroiodophenylated-SRC-specific B cells. (4). All these frequencies, including that for A5A idiotype, are surprisingly high and appear to disagree with frequencies of specific antibodies in the total pool of natural serum Ig. If natural serum Ig constitutes a representative mixture of products of all existing B cells, we would expect 1 in every 2,500 Ig molecules of normal serum to carry the A5A idiotype.

Since the detection limit of our radioimmune assay is in the order of 30 ng of A5A/ml and since the assay is limited by concentrations of nonspecific Ig in the order of 1 mg/ml, we would be able to detect A5A-bearing Ig molecules in normal serum Ig if their frequency would exceed 1 in 30,000. This, however, is not the case. As reported previously (5), the proportion of A5A-bearing Ig molecules in normal Ig is less than 1 in 30,000. This figure is more than an order of magnitude lower than that expected from the frequency of precursor cells.

The discrepancy between these figures can have a number of reasons. We can assume, for example, that the proportion of A5A-bearing molecules in the predominant class of serum immunoglobulin IgG is dramatically smaller than in serum IgM, since in our culture system predominantly IgM-producing precursor cells are estimated (W. Gerhard and F. Melchers, unpublished observations). This would result in a low proportion of A5A idiotype on the total serum Ig even if 1 in 2,500 of the IgM molecules has this idiotype. Examples for a high concentration of an antibody or idiotype in normal IgM in combination with the absence of this antibody from the normal IgG fraction are known for natural anti-phosphorylcholine antibodies (13) and for natural antibodies to haptens such as nitroiodophenyl and nitrophenyl (14, 15). If this were a general phenomenon, we would have to conclude that the IgG pool represents a selection from the IgM precursor repertoire. We should like to state that the discrepancies observed between the serum levels and the precursor frequencies remain a problem that needs further investigation.

Memory was clearly demonstrable in quantitative terms, in the B-cell compartment of Strep A and of anti-A5A idiotype IgG1 sensitized mice as a 10--20-fold increase in the frequency of A5A idiotype-producing cells. This increase is in agreement with the 10–20-fold greater idiotype production in primed mice as compared to normal mice upon Strep A challenge (5, 6, 8). It is interesting to note that the amounts of idiotype produced in primed and unprimed cultures are exactly the same on a per clone basis (discussed below). Thus, it appears that the generation of memory within the LPS-reactive B-cell compartment can be expressed as an augmentation of the number of precursor cells without any change in the capacity of the cells to mature and secrete antibody.

The data obtained on A5A-suppressed cells are somewhat less conclusive. The cells analyzed for these experiments came from mice in which suppression is mediated by suppressor T cells induced by injection of a low dose (0.1 μg) of anti-A5A IgG2 (10). In such mice, it is not clear whether the 20--50-fold reduction in their idiotype response to Strep A challenge is the result of a

reduction of A5A precursor B cells or of the suppression of T-helper cells that cooperate with such B cells. From the idiotype response of suppressed mice in vivo we would expect a much greater reduction in the A5A precursor frequency than the 2.5–3-fold reduction observed here, if suppression and reduction of B precursor cells were correlated (10). It should be mentioned, however, that (*a*) the numbers of suppressed cultures tested were small (Table II) and (*b*) the point in the degression line on which the frequency estimate relies (see Fig. 1) is already in the zone at which nonspecific Ig interferes with the test system. Therefore, our frequency determination for A5A precursor B cells in mice carrying idiotype suppressor T cells is most likely an overestimate, and the reduction of such cells is probably more pronounced. Because of the limitation of the test system, we cannot, with safety, quantitatively estimate to which extent A5A-producing B cells have been reduced in suppressed mice. On the other hand, the data reveal the differential effect of IgG1 and IgG2 anti-idiotype antibodies on idiotype-bearing precursor lymphocytes: whereas IgG1 antibodies expand the precursor pool, there is no such effect associated with IgG2 antibodies (7, 8).

The present results show that memory (and perhaps suppression) in the B-cell compartment can be quantitated as frequencies of specific cells. The observed frequencies of A5A idiotype-producing B cells in normal and primed mice strongly suggests that the LPS-reactive third of all B cells in spleen is representative for all B cells to the extent that it is influenced by priming with antigen and anti-idiotype in parallel with the total pool of specific cells reacting to an antigenic challenge (6–8).

It becomes evident from our experiments with unprimed and particularly with suppressed mice that frequency determinations are not possible for idiotype-producing cells occurring with frequencies lower than one in 3×10^3 as long as the current culture conditions and the current version of the radioimmunoassay are used. Cultures with 1 A5A idiotype-producing B-cell clone contain 3,000 other LPS-reactive B-cell clones, all secreting IgM. Total IgM reach concentrations between 100 μg and 1 mg/ml in the cultures. This concentration of nonspecific IgM interferes in the radioimmunoassay, such that even without the presence of A5A idiotype-bearing antibodies inhibition is observed. Thus, cultures with more than 5×10^3 cells cannot reliably be tested for A5A idiotype. Since also the hemolytic plaque assay for any determinant coupled to SRC is limited by the frequency of SRC-specific B cells, i.e. 1 in 1,000 LPS-reactive cells (4), we have at present no assay to detect any specific B cells which occur with frequencies below 1 in 3×10^3.

Our radioimmune assay, though limited by the interference of normal Ig at high concentration, allows the quantitative determination of idiotype-bearing immunoglobulin molecules in culture supernates. It is very surprising that one clone of A5A idiotype-producing B cells within 10^2–2×10^3 clones secreting IgM with other idiotypes secretes such a constant amount of A5A idiotype (30 ng). This appears to be a property of the culture system and not due to detection limits in the radioimmunoassay (see Materials and Methods). It is furthermore supported by data obtained for parainfluenza- and influenza virus-specific antibodies in another radioimmunoassay, in which one clone of B cells-producing specific antibody, mainly of IgM class, within 10^2–10^3 other B-cell clones

clones have been found to secrete again 30 ng-specific antibody (W. Gerhard and F. Melchers, unpublished observations). The fixed amount of secreted Ig per clone of growing B cells is all the more surprising, since culture media were not collected at a fixed time, but at various times between 10 and 14 days of culture. Although we know that LPS-stimulated clones of B cells divide quite regularly every 18 h, at least within the first 5 days of culture (2), we also know that they cease to grow after 1 wk of culture and reach a plateau level of 30 ng/clone between 7 and 8 days of culture. The regulatory factor(s) which cause(s) this strict limitation in growth and secretion remain(s) to be investigated.

It should be mentioned that the figure of 30 ng/clone is essentially reasonable as it would require 1 day of secretion of 500 plasma cells each producing 2,000 molecules/s. This number of cells is generated after eight divisions of a single precursor cell within 6–7 days.

We observed a high association between the presence of the A5A idiotype and the specificity of the antibody for group A-CHO. Out of two clones producing antibody with A5A idiotype, one produces antibody which also binds to Strep A organisms and presumably has specificity to A-CHO. This high association can be explained by the specificity of the anti-A5A antiserum used in these studies. The majority of its antibodies (~90%) reacted with idiotype determinants generated by the combination of heavy and light chain, while a minority reacted with heavy chain determinants alone (16). Thus, this antiserum recognizes a particular V_H-V_L combination defining an A-CHO-binding site.

It is, however, surprising that even after priming with the antigen, Strep A-CHO, resulting in a 10-fold increase in the number of idiotype-bearing B-cell precursors, one half of all idiotype-producing clones still lack specificity for A-CHO. Such Ig molecules are comparable to the idiotype-bearing, non-antigen-reactive Ig molecules described by Oudin and Cazenave (17).

Priming of nonantigen-recognizing, but idiotype-positive B cells upon antigenic challenge is predicted by Jerne in his network hypothesis (18). It assumes that two or more Ig molecules (on two or more cells) which are structurally different in their combining site and, therefore, recognize different epitopes, may bear similar idiotypic determinants and, therefore, be under control of overlapping anti-idiotypic sets (cells). An initial increase in the number of antigen-reactive Ig molecules (i.e. numbers of cells) bearing the idiotype, effected by antigen exposure of the antigen-binding subset of idiotype-bearing molecules (cells) will result in derepression from anti-idiotypic action of the entire idiotype-bearing population and, concomitantly, in equal increases in the number of all other idiotype-bearing molecules (cells) independent of their antigen reactivity.

Summary

The absolute frequencies of B cells-producing A5A idiotype have been determined in vitro by limiting dilution analysis in a culture system in which every LPS-reactive B cell grows into a clone of IgM-secreting cells. Spleen cells from normal A/J mice contain 1 A5A-idiotype-producing B-cell precursor in 2.5 $\times 10^3$ LPS-reactive B cells. Approximately a 10–20-fold increase in frequencies

of precursor cells results from antigen priming with Strep A-CHO (1 in 2.8 × 10^2) or from sensitization with IgG1 anti-A5A idiotype (1 in 1.3 × 10^2). Injection of IgG2 anti-A5A idiotype which has been shown to suppress A5A idiotype in vivo results in only a marginal and maybe insignificant decrease in precursor frequencies (1 in 6.7 × 10^3). On the other hand, priming does not result in a detectable qualitative difference in the specific precursor cells, since each clone of B cells secretes 30 ng of A5A-bearing Ig within 8 days of culture, regardless of being unprimed or primed.

Nearly half of all A5A idiotype-producing clones, both from unprimed as well as from primed mice, show antigen specificity in binding A-CHO. Priming by antigen, therefore, also results in a 10-fold increase in the frequency of idiotype positive B cells without antigen specificity. This result is a prediction of the network hypothesis.

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