

# FREQUENCIES OF MITOGEN-REACTIVE B CELLS IN THE MOUSE

## I. Distribution in Different Lymphoid Organs from Different Inbred Strains of Mice at Different Ages

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We have recently been able to improve the conditions for *in vitro* lymphocyte cultures such that every growth-inducible murine B cell, in the presence of a B-cell mitogen, will initiate growth and maturation to IgM-, IgG-, and IgA-secreting plaque-forming cells (PFC) (1, 2).<sup>1</sup> This was achieved mainly by adding 2-mercaptoethanol (3), a growth-supporting fetal calf serum (4), growth-supporting thymus cells (2), and a B-cell mitogen (4-6) to the cultures. The presence of thymus cells, in particular, yielded a large increase in the number of growth-initiating B cells. We found that every dividing B cell within a clone, stimulated by mitogen, secreted Ig (2). A modified hemolytic plaque assay with protein A-coated sheep erythrocytes (SRC) and anti-Ig antisera as developing antibodies was employed to detect every Ig-secreting cell as a plaque (7). These culture conditions and the plaque assay for the detection and enumeration of all stimulated, secreting B-cell clones made it possible to do limiting dilution analyses of the number of bacterial lipopolysaccharide (LPS)-reactive B cells. Every third B cell in these spleen cell preparations of 6- to 8-wk old C3H/Tif mice proved to be LPS-reactive yielding a clone of IgM-secreting PFC (2).

The capacity of a given lymphocyte population to respond to a B-cell mitogen can, therefore, be assessed by limiting dilution analyses *in vitro* and can be expressed as frequencies of reactive B cells. In this paper we determine the frequencies of mitogen-reactive B cells yielding an IgM-PFC response mainly to the mitogen LPS (5) and in some cases also to the mitogen lipoprotein (6) in spleen, lymph node, bone marrow, thymus, thoracic duct, and peripheral blood of several inbred strains of mice at different ages.

### Materials and Methods

*Mice.* C3H/Tif/BOM- and BALB/c/BOM nu/nu-mice were obtained from Gr. Bomholtgaard, Ry, Denmark. C3H/HeJ/Fül-, BALB/c/Fül-, C57BL/6J/Fül-; DBA/2J/Fül-, CBA/Fül-, A/J/Fül-, SJL/Fül- and C57BL/6J/Fül × DBA/2J/Fül-mice were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland. Their age is indicated in the Results section.

<sup>1</sup> *Abbreviations used in this paper:* B, bone marrow-derived, bursa equivalent; BSS, balanced salt solution; Ig, immunoglobulin; LPS, bacterial lipopolysaccharides; PFC, plaque-forming cell; SRC, sheep erythrocytes; v, variable.

*Cells.* All cells were prepared at room temperature under sterile conditions. Spleen, mesenteric lymph node, and thymus cells were prepared by passing the cells of these organs through a 200-mesh stainless steel screen, in balanced salt solution (BSS) containing in most cases 10% fetal calf serum. Bone marrow cells were extracted from the femurs by cutting open the ends of the femurs, attaching a 25 gauge-needle to one end, and pushing the cells out at the other end with BSS containing 10% fetal calf serum. The cell clumps were then dissociated by pipetting up and down in a 10-ml plastic pipette (Falcon no. 7551, Falcon Plastics, Oxnard, Calif.) 10 times. Thoracic duct cells were obtained by overnight drainage of the thoracic duct following the method of Sprent (8). Peripheral blood lymphocytes were obtained from heparinized (Liquemin, Roche, Basel, Switzerland) blood. Erythrocytes were, at least to a larger part, removed by lysis for 3 min at 37°C in 0.83%  $\text{NH}_4\text{Cl}$ . From all cell preparations possible cell clumps were allowed to settle for 5 min at 1 g. All cell suspensions were then washed twice over a layer of fetal calf serum (centrifugation for 10 min at 200 g) and finally resuspended in RPMI 1640 tissue culture medium (Grand Island Biological Co., Grand Island, N. Y.) (see below). Viable cells were determined by the trypan-blue exclusion method. Nucleated cells were counted in Türk's solution. Viabilities ranged for spleen between 75 and 90%, for lymph node between 85 and 95%, for all other cell preparations above 90%.

Whenever murine thymus cells were used as growth-supporting cells in culture (2) they were obtained from 4-wk old syngeneic or allogeneic mice and passed twice through columns of nylon wool (Fenwal Laboratories, Bern, Switzerland) (9). This double passage through nylon wool was mandatory to remove contaminating LPS-reactive B cells which developed into IgM-secreting PFC if they were left in the thymus cell preparations (12, see also Results with thymus cells). Recently we have found (Lernhardt, W., and F. Melchers. Manuscript in preparation) that xenogeneic thymus cells, i.e. from 4- to 6-wk old Lewis-strain rats (Institut für Biologisch-Medizinische Forschung AG), support the growth of murine B cells just as efficiently as murine thymus cells. This offers the advantage that more cells are readily available. Thymus cells from rat also do not have to be passed through nylon wool, since rat thymus either does not contain LPS-reactive B cells developing into IgM-secreting PFC, or, if they do, these PFC are not detected in the protein A-SRC plaque assay with the mouse Ig-specific antibodies. We indicate in the Results section which source of thymus cells has been used as growth support in the experiments.

*Culture Medium.* RPMI 1640 medium was used fresh (not older than 2 mo) from Microbiological Associates, Bethesda, Md., or from Bio-Cult, Irvine, Scotland. Glutamine (2 mM), penicillin, and streptomycin (5,000 IU/ml each), HEPES-buffer (10 mM) pH 7.3; 2-mercaptoethanol ( $5 \times 10^{-8}$  M), and fetal calf serum (10%), batch K 255701 D (Bio-Cult), specifically tested for growth-supporting properties and for low or undetectable endogenous mitogenic activities (4), were added. Cells were cultured in 5-ml plastic tubes in 0.2-ml aliquots (Falcon no. 2058, Falcon Plastics). At the termination of culture, 5 ml cold BSS was added, the cells were spun for 10 min at 200 g, and resuspended in appropriate volumes of cold BSS for plaque assay.

*Mitogens.* LPS-S (EDTEN 18735 and S495/188049) 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 in culture at 50  $\mu\text{g}/\text{ml}$  (10). *Escherichia coli* lipoprotein (6), a gift of Dr. V. Braun, Mikrobiologie II, Universität Tübingen, Tübingen, West-Germany, was used at 2  $\mu\text{g}/\text{ml}$ .

*Plaque Assay for Ig-Secreting Cells.* A modified hemolytic plaque assay with protein A-coated SRC and mouse Ig-specific antisera as developing antibodies in the presence of complement was employed to detect and enumerate Ig-secreting cells (7). IgM-secreting PFC were scored throughout the experiments reported in this paper. The IgM-specific antisera used in the plaque assay were raised by repeated injections of 0.5–1 mg purified MOPC 104 E 19S IgM (11) in Freund's incomplete adjuvants. The antisera detected  $\mu\text{H}$  and  $\lambda\text{L}$  chains-, but not  $\gamma\text{H}$ - or  $\alpha\text{H}$ - or  $\kappa\text{L}$ -chain-secreting cells as tested in plaque assays with the corresponding myeloma tumor cell suspensions. Protein A used in coating of SRC was obtained from Dr. H. Wigzell, Biomedicum, University of Uppsala, Uppsala, Sweden. Complement, diluted appropriately with BSS, was from Bio-Cult.

In the experiments where IgG-secreting PFC were scored, a mixture of rabbit antibodies raised against the mouse myeloma proteins MOPC 21 (7S IgG<sub>1</sub>;  $\kappa, \gamma_1$ ) and Adj-PC-5 (7S IgG<sub>2</sub>;  $\kappa, \gamma_2$ ) was used to develop the plaques.

## Results

In all experiments reported here the frequency of mitogen reactive B cells was

determined by measuring the number of responding cultures, i.e. generating an IgM-secreting PFC response, at dilutions limiting the number of reactive B cells to around one. According to Poisson's distribution one reactive B cell is present in that number of cultured cells which will let 63% of all cultures grow into IgM-secreting PFC.

The number of cells limiting the mitogen-reactive B cells in the different cell preparations to approximately one per culture, was first determined in screening experiments by 3.3-fold-serial dilutions from  $1 \times 10^6$  cells to one cell per culture. All cultures were kept in medium containing the constant number of  $3 \times 10^6$  thymus cells per milliliter (see Materials and Methods). 10 cultures were assayed at each cell dilution. Since the doubling time of mitogen-activated B cells is 18 h, cultures yielding more than 15 IgM-secreting PFC at day 5 were scored as positive, responding cultures.

In the range of cell concentrations where the IgM-PFC responses were found to become fluctuating, indicating that the reactive B cells had become limiting, more narrow differences of cell concentrations were tested. Usually, 40 cultures each were set up at 5–10 cell concentrations within a range yielding between 10 and 90% nonresponding cultures. Again, cultures which yielded more than 15 IgM-PFC at day 5 were scored as positive, responding cultures. In most experiments a discontinuous distribution of the number of IgM-PFC per culture was found in cultures at cell concentrations where the responses were fluctuating. Many cultures had clone sizes of IgM-PFC around 60 which were of the size expected from our previous results (2). The logarithm of the fraction of nonresponding cultures obtained with different cell concentrations was then plotted against the corresponding cell numbers in culture. The number of cultured cells containing one mitogen-reactive B cell was extrapolated from these data as that number of cells with which 37% of all cultures did not respond by an IgM-PFC response. If a linear degression of the logarithm of the fraction of nonresponding cultures was found with increasing numbers of cells in the cultures, this was an indication that the responding B cells had been limiting in the cultures.

*Frequencies of LPS- and of Lipoprotein-Reactive B Cells in Spleen of Different Inbred Strains of Mice.* Spleen cells of 6- to 8-wk old mice of inbred strains listed in Table I were diluted in medium containing  $3 \times 10^6$  thymus cells per ml to spleen cell concentrations limiting the number of responding B cells to around one and grown in the presence of LPS. Spleen cells from five of these inbred strains of mice were also grown in the presence of another B-cell mitogen, *E. coli* lipoprotein. Fig. 1 shows two representative experiments demonstrating that mitogen-reactive B cells were limiting in these cultures. Frequencies of mitogen-reactive B cells were calculated from the number of cultured cells which yielded in 63% of all cultures an IgM-PFC response, and, thus, contained one mitogen-reactive B cell. The frequency determinations for LPS-reactive and for lipoprotein-reactive B cells in spleen are summarized in Table I.

LPS and lipoprotein-reactive B cells were found in high frequencies in spleen of most inbred strains of mice which we tested, i.e. one reactive B cell in between 6 and 20 spleen cells. If we assume that in all these spleen cell preparations, including those of BALB/c nu/nu origin (12) half of the cells are B cells, then one mitogen-reactive cell was found among three to seven splenic B cells. Spleen cells from normal, T-cell-containing and from nu/nu BALB/c-mice, lacking

TABLE I  
Frequencies of LPS- and of Lipoprotein-Reactive B Cells in Spleen of Several Inbred Strains of Mice

| Mouse strain        | Source of growth-supporting thymus cells | Frequency* (mitogen-reactive B cells in number of nucleated cells) |             |             |
|---------------------|--|--|-------------|-------------|
|                     |  | Mitogen: LPS   | Lipoprotein | None        |
| BALB/c              | BALB/c                                   | 1 in 20  | 1 in 18     | ND          |
| BALB/c nu/nu        | BALB/c                                   | 1 in 16  | 1 in 16     | ND          |
| C57BL/6J            | Lewis rat                                | 1 in 6   | ND          | ND          |
| DBA/2J              | Lewis rat                                | 1 in 10  | ND          | ND          |
| C57BL/6J × DBA/2JF1 | Lewis rat                                | 1 in 10  | ND          | ND          |
| CBA                 | Lewis rat                                | 1 in 4   | ND          | ND          |
| A/J                 | Lewis rat                                | 1 in 6   | ND          | ND          |
| C3H/Tif             | C3H/Tif                                  | 1 in 6   | 1 in 8      | 1 in 30,000 |
| C3H/HeJ             | Lewis rat                                | 1 in 10,000  | 1 in 6      | 1 in 10,000 |
| SJL                 | Lewis rat                                | 1 in 60  | 1 in 50     | ND          |

\* Determined in plots of the logarithm of the fraction of nonresponding cultures against the number of cultured cells as the number of cultured cells yielding 37% nonresponding cultures (see Fig. 1). ND, not done.

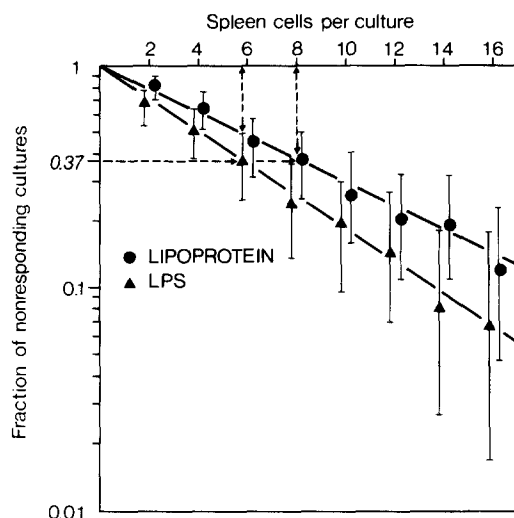


FIG. 1. Titration of LPS- and of lipoprotein-reactive B cells. Abscissa indicate the number of input spleen cells of C3H/Tif-mice (6–8 wk of age) per culture in the presence of either 50  $\mu\text{g/ml}$  LPS ( $\blacktriangle$ ) or 2  $\mu\text{g/ml}$  lipoprotein ( $\bullet$ ) and a constant number of rat thymus cells ( $3 \times 10^6$  cells/ml). The assays of IgM-secreting PFC were done at day 5 of culture. The dotted lines point to the number of spleen cells in culture with which 37% of all cultures did not yield a response, i.e. which, according to Poisson's distribution, contained one mitogen-reactive B cell. Vertical bars represent 95% confidence limits.

mature T cells, both showed very similar frequencies of LPS- and of lipoprotein-reactive B cells. Only SJL mice showed markedly decreased frequencies of LPS- and of lipoprotein-reactive B cells. C3H/HeJ spleen cells, genetically nonresponsive to LPS but responsive to lipoprotein (6, 13, 14), did not show any number of LPS-reactive B cells which could be distinguished above the background of B

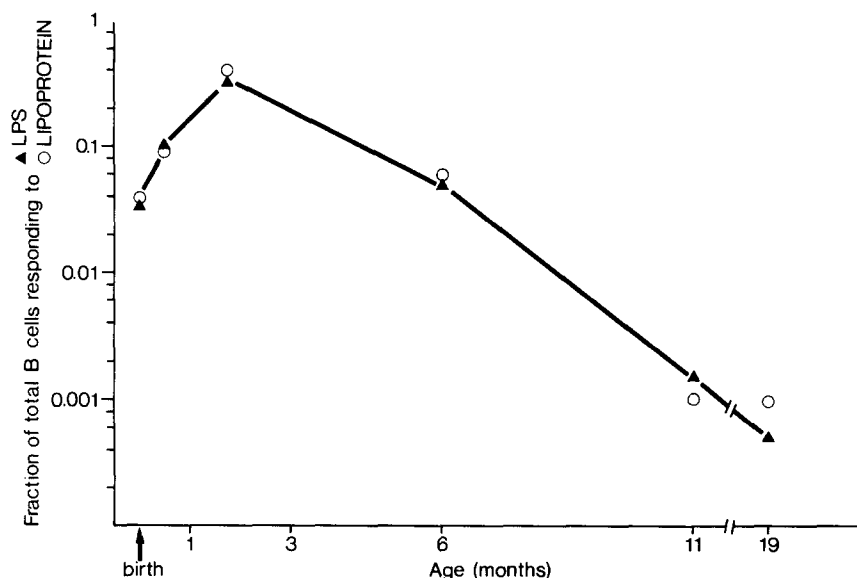


FIG. 2. Frequencies of LPS- and of lipoprotein-reactive B cells in spleen of C3H/Tif-mice at different ages. Frequency determinations were done as indicated in the text (see also Fig. 1). ▲, LPS; ○, lipoprotein.

cells in mitogen-free cultures. These B cells in mitogen-free cultures most probably respond to B-cell mitogens present in fetal calf serum (15) used in the culture medium (see Materials and Methods). C3H/HeJ spleen cells contain one lipoprotein-reactive B cell in six spleen cells, the normal complement of responsive cells, expected from their genetically determined ability to respond to lipoprotein.

*Frequencies of LPS- and of Lipoprotein-Reactive B Cells in Spleen of C3H/Tif Mice of Different Age.* The frequencies of LPS- and of lipoprotein-reactive B cells were determined in spleen cells of C3H/Tif mice at different ages. Newborn-, 2-wk, 6- to 8-wk, 6-, 11-, and 19-mo old mice were compared. Fig. 2 summarizes the results. The highest frequencies of LPS- and of lipoprotein-reactive B cells were found in 6- to 8-wk old mice. The relative frequencies of LPS- and of lipoprotein-reactive cells did not change significantly at any age. At birth, 1 reactive cell was found in 30–50 spleen cells. Old mice (11 mo of age or older) had less than 5% of the number of mitogen-reactive B cells found at 6–8 wk of age. In all experiments the reactive B cells were limiting in the cultures.

*Frequencies of LPS-Reactive B Cells in Different Lymphoid Organs.* Frequencies of LPS-reactive cells were determined in mesenteric lymph node, bone marrow, thymus, thoracic duct, and peripheral blood cells of 6- to 8-wk old mice of inbred strains of mice listed in Table II. The LPS-reactive B cells were limiting in all experiments. Mesenteric lymph nodes had numbers of LPS-reactive cells very similar to spleen (see Table I). In contrast to the other cell sources, bone marrow cells are heterogeneous in their response to LPS. Small cells of bone marrow yield an immediate IgM-PFC response, usually measured at day 5 of culture. Large cells of bone marrow develop an IgM-PFC response which is barely detectable at day 5 and which is measured at day 8–10 of culture

TABLE II  
*Frequencies of LPS-Reactive B Cells in Bone Marrow, Thymus, Thoracic Duct, and Peripheral Blood*

| Cell source            | Mouse strain        | Source of growth-supporting thymus cells | Frequency*<br>(LPS-reactive B cells in number of nucleated cells) |
|------------------------|---------------------|--|---|
| Mesenteric lymph nodes | BALB/c              | BALB/c                                   | 1 in 10   |
|                        | C3H/Tif             | Lewis rat                                | 1 in 6  |
| Bone marrow            | BALB/c              | BALB/c                                   | 1 in 200  |
|                        | C3H/Tif             | Lewis rat                                | 1 in 50   |
| Thymus                 | C3H/Tif             | Same, not passed over nylon wool         | 1 in 10 <sup>5</sup>  |
|                        | C57BL/6J × DBA/2JF1 | Same, not passed over nylon wool         | 1 in 10 <sup>5</sup>  |
| Thoracic duct          | BALB/c nu/nu        | BALB/c                                   | 1 in 10   |
|                        | C3H/Tif             | Lewis rat                                | 1 in 40   |
| Peripheral blood       | C3H/Tif             | Lewis rat                                | 1 in 20   |
|                        | C57BL/6J × DBA/2JF1 | C57BL/6J × DBA/2JF1                      | 1 in 20   |

\* Determined in plots of the logarithm of the fraction of nonresponding cultures against the number of cultured cells as the number of cultured cells yielding 37% nonresponding cultures (see Fig. 1).

(Phillips, R. A., and F. Melchers. Unpublished observations; Phillips, R. A. Personal communication). The frequencies of LPS-reactive cells in bone marrow given in Table II are for the former cell type only. Thymus cell preparations contained between 1 and 2 LPS-reactive B cells in 10<sup>5</sup> cells, even when special care was taken to avoid contamination by adjacent lymph nodes and by blood. As pointed out in the Materials and Methods section, thymus cells have to be passed over nylon wool to remove reactive B cells whenever they are to be used as growth-supporting cells. Thoracic duct and peripheral blood cells contained similar numbers of LPS-reactive B cells. These experiments, for the first time, show that murine peripheral blood B lymphocytes can respond to stimulation by LPS with growth and maturation to Ig-secreting PFC.

All cell preparations listed in Table II could be stimulated by LPS to IgG secretion. The peak of IgG-secreting PFC occurred between days 7 and 10 of culture.

### Discussion

A series of inbred strains of mice, known to be responsive to the B-cell mitogens LPS and lipoprotein, contain in their spleen a high number of B cells reactive to these mitogens. Our frequency analyses indicate that this number is 1 mitogen-reactive B cell among 3-10 B cells. These high frequencies clearly indicate that B-cell mitogens must stimulate B cells directly, circumventing the binding to surface Ig (5, 16). The stimulation of mitogen-reactive B cells,

therefore, results in polyclonal growth and maturation of B cells, each clone secreting one set of variable (v)-regions on Ig molecules specifying their antigen-binding capacity, within the large repertoire of all v-region sets expressed by B cells. The capacity of a B cell to grow and to expand the expression of its Ig by secretion depends, therefore, on the capacity of this cell to be mitogen reactive. Mitogen reactivity is most probably a function of the expression of mitogen receptors on the surface of B cells. These mitogen receptors must be distinct from Ig molecules, but may be functionally and structurally linked to them (17).

Our results show that the proportion of LPS- and of lipoprotein-reactive B cells within the total population of B cells changes with age. At 6–8 wk of age a large proportion of all B cells, but not all of them, are reactive to LPS and to lipoprotein. This suggests that other B-cell mitogens exist which stimulate those B-cell populations which are not stimulated by LPS or lipoprotein. Candidates for these other B-cell mitogens are other external mitogens as well as internal mitogens (factors) produced by either T cells or macrophages' (18, 19). One B cell may possess more than one mitogen receptor (20). On the other hand, it also appears likely that mitogen-reactive B cells form only partially cross-reactive subpopulations of B cells (21). We are currently investigating how many of the LPS-reactive B cells are also lipoprotein-reactive, and how many of them belong to noncross-reacting populations of B cells.

We assume that each of these different mitogen-reactive B-cell subpopulations express the whole repertoire of v-region sets on Ig molecules. Mitogens can, therefore, be used to analyze the total v-region repertoire of B cells by stimulating all mitogen-reactive cells to Ig secretion. In this way, we have analyzed the repertoire of v regions on Ig molecules which are detected by antigen-specific assays for B-cell Ig (Succeeding publication).

Frequency determinations of reactive cells *in vitro* can be taken as quantitative analyses of the capacity of a given cell population to react to a stimulating agent. C3H/HeJ-mice are genetically nonresponsive to LPS, but normally responsive to lipoprotein (6, 13, 14). Consequently, we found no LPS-reactive B cells detectable above background while normal numbers of lipoprotein-reactive cells were present. The background activities of growing B cells in our cultures without mitogen are most probably due to B-cell mitogens in fetal calf serum (15). Our frequency analyses show a marked lower frequency for both LPS- and for lipoprotein-reactive B cells in SJL mice.

We have also determined the frequency of mitogen-reactive B cells in different lymphoid organs of the mouse. LPS-reactive B cells were known to exist in mesenteric lymph nodes, thoracic duct, and bone marrow. In thymus and peripheral blood, LPS-reactive B cells yielding an Ig-secreting PFC response had not been found previously with less sensitive methods of *in vitro* culture. Thymus cells, not passed over nylon wool, contained one to two LPS-reactive B cells in  $10^5$  cells. This certainly constitutes the minimal estimate of the number of B cells in thymus, since we do not know at present how many B cells with other mitogen reactivities reside in thymus. B-cell contamination in thymus is, therefore, so extensive that it must be considered to interfere in all experiments where thymus cells are assayed for the presence, the synthesis and the biological activities of B-cell-like Ig (22, 23).

Experiments of B-lymphocyte stimulation with peripheral blood lymphocytes of mice were never feasible since previous *in vitro* culture conditions needed far more than  $10^6$  lymphocytes for an experiment. Our improved culture conditions for B cells do not need more than  $10^3$  peripheral blood cells to detect a response of IgM- and IgG-secreting PFC. The frequency of LPS-reactive cells was 1 in 20–30 peripheral blood cells and was, thus, as high as in spleen, considering that a large fraction of all peripheral blood cells are not B lymphocytes. Since peripheral blood B cells can now respond *in vitro* by growth and maturation to Ig secretion, it becomes possible to screen mitogen reactivities in living mice. This is of obvious importance in genetic experiments.

### Summary

Frequencies of mitogen-reactive B cells have been determined *in vitro* under culture conditions which allow every growth-inducible B cell to grow and mature into a clone of Ig-secreting PFC.

The frequencies of LPS-reactive B cells in the spleen of 6- to 8-wk old mice were between 1 in 3 and 1 in 10 splenic B cells from the following inbred strains of mice: C3H/Tif; BALB/c; BALB/c nu/nu; C57BL/6J; DBA/2J; C57BL/6J  $\times$  DBA/2<sub>F</sub><sub>1</sub>; and CBA and A/J. Very similar frequencies are found for lipoprotein-reactive B cells in BALB/c, BALB/c nu/nu, C3H/Tif, and C3H/HeJ mice. No LPS-reactive cells but normal frequencies of lipoprotein-reactive cells were found in C3H/HeJ mice, genetically nonreactive to LPS. SJL mice had significantly lower frequencies of LPS- and of lipoprotein-reactive B cells (1 in approximately 30 B cells).

The number of LPS- and of lipoprotein-reactive B cells in spleen was dependent upon the age of the mouse. Newborn spleen contained approximately 10% of the number of reactive cells found at 6- to 8-wk of age. From there the frequencies declined again to drop below 5% of the maximal number at ages beyond 11 mo.

LPS-reactive B cells yielding IgM- and IgG-PFC responses could be found in mesenteric lymph nodes, bone marrow, thymus, thoracic duct, and peripheral blood of 6- to 8-wk old mice. Their frequencies were one in three to five lymph node cells, 1 in 50 to 100 bone marrow cells, one in  $10^5$  thymus cells, and 1 in 20 to 40 thoracic duct or peripheral blood cells.

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