DEFECTIVE COLONY FORMATION BY B LYMPHOCYTES FROM CBA/N AND C3H/HeJ MICE*

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Since the adaptation of semisolid culture methodology to the cloning of mitogen-stimulated lymphocytes (1), progress has been rapid in defining culture variables which affect colony formation and characterization of the cells that proliferate. It is now apparent that lymphocyte colony formation is strictly mitogen dependent and that most B cells which clone bear IgM and Ia receptors; are widely distributed anatomically; are dependent on macrophages in addition to 2-mercaptoethanol (2ME); and have a sensitivity to divalent anti-immunoglobulin (Ig) or anti-Ia antibodies, which is characteristic of mature B-cell populations (2-5). Our understanding of the cells that form colonies is extended by the present studies of two well-characterized, genetically controlled, murine immunodeficiencies. Extensive studies with CBA/N mice reveal their X chromosome-linked inability to mount normal immune responses to thymus-independent antigens and poor in vitro responsiveness to B-cell mitogens (6-9). Associated with this defect is a reduced number of splenic B cells, low serum IgM levels, an abnormally high ratio of B-cell surface IgM relative to putative IgD, and an immature pattern of cell surface Ig-density distribution (10-13). Evidence is presented here that homozygous CBA/N mice lack functional colony-forming B cells entirely and confirms that this defect is intrinsic to B cells. C3H/HeJ strain mice have been well studied for their genetically controlled hyporesponsiveness to certain forms of lipopolysaccharide (LPS) (14-18). They were found to have extremely low but detectable numbers of colony-forming B cells. These results suggest a selectivity of the cloning assay for a functional subpopulation of B cells and further define these animal models of immune-deficiency disease.

Materials and Methods

Animals. CBA/N mice were raised under specific pathogen-free conditions at Sloan-Kettering Institute from a breeding stock obtained from the N.I.H., Bethesda, Md. or were obtained as

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Abbreviations used in this paper: AM, agar-derived mitogens; B1, B lymphocytes which are developmentally thymus-independent and functionally independent of thymus-derived cells; B2, B cells which are functionally dependent on cooperative interactions with thymus-derived cells; CFU-c; granulocyte-macrophage progenitors; CFU-s; multipotential stem cells; Con A, concanavalin A; CR, receptors for the third component of complement; DS, dextran sulfate; FCS, fetal bovine serum; ¹³¹IUDR, ¹³¹I-iododeoxyuridine; LPS, lipopolysaccharide; 2 ME, 2-mercaptoethanol; mφ, adherent macrophages; PBS, phosphate-buffered saline.
adults from Altick Associates, Hudson, Wis. CBA/Cum (obtained from Cumberland View Farms, Clinton, Tenn.) or CBA/H-TtTt mice (maintained at Sloan-Kettering from a stock derived from The Jackson Laboratories, Bar Harbor, Maine) of either sex were used as controls for experiments involving CBA/N mice. Macrophages for some experiments were obtained from (C57BL/6 x DBA/2)F1, mice (Cumberland View Farms). Initial experiments with C3H mice involved C3H/StCR (Charles River Breeding Laboratories, Wilmington, Mass.) and wild-type C3H/HeJ mice from a colony derived from The Jackson Laboratories and on which the W" mutation is maintained. Later experiments were done comparing C3H/HeJ and C3HeB/FeJ mice obtained as adults from The Jackson Laboratories.

Cell Cultures. Semisolid and liquid cell cultures as well as granulocyte-macrophage progenitor (CFU-c) cell assays were performed essentially as described before (2) with the following modifications. Conventional liquid mitogen cultures and clonal assays were incubated in 7% CO2 in fully humidified air. A single batch of fetal bovine serum (FCS) (lot no. 1027; Flow Laboratories, Inc., Rockville, Md.) was used throughout. This particular serum supports slightly fewer B-cell colonies in agar mitogen-stimulated cultures than some other batches which we tested, but was selected because it caused an acceptably low background stimulation in liquid cultures. Agar-supported cultures were potentiated by the addition of LPS (10 μg/ml culture), sheep red blood cells (SRBC) (0.1 ml of a 5% solution of washed SRBC obtained from Flow Laboratories, Inc.), macrophage feeder layers, or combinations of these. Feeder layers were prepared by allowing 10⁶ thioglycolate-activated peritoneal macrophages to adhere to Lux Contur culture dishes (Lux Scientific Corp., Thousand Oaks, Calif.) which were subsequently washed and overlaid with a 1 ml layer of 0.5% agar containing complete medium and 1.4 × 10⁻⁷ M indomethacin. A full discussion of this procedure and its effects will be presented elsewhere. Briefly, this insures an adequate supply of diffusible macrophage-derived factors, prevents prostaglandin-mediated inhibition, which results from an overabundance of macrophages, and results in colony formation which is a linear function of the number of B cells cultured. Mitogens used for stimulating liquid cultures were LPS from Salmonella typhosa W9001 (Difco Laboratories, Detroit, Mich.), 5 μg/culture; agar-derived mitogens (AM) prepared as described (2), 25 μg/culture; dextran sulfate (DS) (mol wt 500,000; Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), 20 μg/culture; and concanavalin A (Con A) (Pharmacia Fine Chemicals, Inc.), 5 μg/culture. B-cell differentiation was assessed after 3-5 days of incubation with LPS using the same culture conditions as for stimulation or in low-cell density liquid cultures prepared according to the method of Kearney and Lawton (19). Cells were harvested from replicate cultures, pooled, evaluated by phase-contrast microscopy, and slides prepared with a cytocentrifuge. These were fixed and stained for the presence of cytoplasmic immunoglobulin with rhodamine-labeled (Fab')2 fragments of anti-μ antibodies described elsewhere (3).

Titration of Serum Inhibitor Levels. Individual samples of blood were collected from the tail, allowed to clot at room temperature, and the serum was separated. To 1 ml semisolid cultures of 10⁶ CBA/Cum lymph node cells was added 0.1 ml of phosphate-buffered saline (PBS) or serum dilutions. Colonies were scored 5 days later, and the dilution required to reduce colony numbers by half determined.

Detection of Multipotential Stem Cells. The incidence of multipotential stem cells (CFU-s) in bone marrow cell suspensions was determined by injecting 5 × 10⁴ cells into lethally irradiated (850 R) CBA/Cum mice and enumerating macroscopic colonies on the surface of the Bouin's fixed spleens 8 days later.

Results

Inability of CBA/N B Cells to Form Colonies. When lymphoid cell suspensions from the spleen, lymph nodes, or peripheral blood of adult homozygous CBA/N mice were cultured in agar, no B-cell colonies were formed. In hundreds of such cultures initiated with up to 10⁶ lymphocytes per culture, only one possible B-cell colony was observed. Attempts were made to induce colony

2 Kurland, J. I., P. W. Kincade, and M. A. S. Moore. Regulation of B lymphocyte clonal proliferation by diffusible macrophage-derived factors. Manuscript in preparation.
### Table I

**Inability of CBA/N B Cells to Form Colonies**

| Cells cultured                  | Potentiated by: | Colonies/culture ± SD |
|---------------------------------|-----------------|------------------------|
| CBA/H-T6 spleen                 | PBS             | 49 ± 7                 |
|                                 | LPS             | 245 ± 49               |
|                                 | SRBC            | 386 ± 40               |
|                                 | LPS + SRBC      | 482 ± 55               |
|                                 | Mφ              | 262 ± 61               |
|                                 | Mφ + SRBC       | >500                   |
|                                 | Mφ + LPS        | >500                   |
| CBA/H-T6 lymph node             | PBS             | 4 ± 2                  |
|                                 | LPS             | 76 ± 20                |
|                                 | SRBC            | 195 ± 32               |
|                                 | LPS + SRBC      | 287 ± 55               |
|                                 | Mφ              | 135 ± 37               |
|                                 | Mφ + LPS        | 196 ± 12               |
| CBA/N spleen                    | PBS             | 0                      |
|                                 | LPS             | 0                      |
|                                 | SRBC            | 0                      |
|                                 | LPS + SRBC      | 0                      |
|                                 | Mφ              | 0                      |
|                                 | Mφ + SRBC       | 0                      |
|                                 | Mφ + LPS        | 0                      |
|                                 | Mφ + SRBC + LPS | 0                      |
| CBA/N lymph node                | PBS             | 0                      |
|                                 | LPS             | 0                      |
|                                 | SRBC            | 0                      |
|                                 | LPS + SRBC      | 0                      |
|                                 | Mφ              | 0                      |
|                                 | Mφ + SRBC       | 0                      |
|                                 | Mφ + LPS        | 0                      |
|                                 | Mφ + SRBC + LPS | 0                      |

All cultures were initiated with 5 x 10⁴ nucleated cells alone or potentiated by the addition of LPS, SRBC, macrophages (Mφ), or combinations of these as described in the Materials and Methods. B-cell colonies were scored after 5 days culture.

Formations by addition of LPS, SRBC, or macrophages to the cultures. These agents potentiate size and number of colonies and improve the linearity of the assay for responding cell populations (references 1, 2, and 4; and J. I. Kurland, P. W. Kincade, and M. A. S. Moore, unpublished observations). Representative experiments are shown in Table I. CBA/N B cells did not form colonies under any circumstances. When high numbers of spleen cells were cultured, some spontaneous granulocyte colonies were observed, and since the macrophage feeders can elaborate colony-stimulating activity, granulocyte-macrophage colonies were frequently observed in cultures potentiated in this way. These were easily distinguishable from B-cell colonies which are composed of less refractile, smaller appearing cells. If any B cells capable of colony formation are present in CBA/N mice, their frequency must be extremely low.
TABLE II

Colony Formation by Mixtures of CBA/N and Normal B Cells

| Exp. | CBA/N | Lymph node (10⁶) | Spleen (10⁵) | CBA/H-T₆T₆ Lymph node (5 × 10⁴) | PBS | LPS | Mø | 10⁴ CBA/N spleen | 5 × 10⁴ CBA/N spleen | 10⁵ CBA/N spleen |
|------|-------|-----------------|--------------|-------------------------------|-----|-----|-----|----------------|-------------------|------------------|
| I    |       | (10⁶)*          | 0†           | 60 ± 6                         | 21  | 112 | 169 | 32 ± 5         | 28 ± 11           | 31 ± 3           |
|      |       | (10⁵)           | 164 ± 14     |                               |     |     |     | 10⁴ CBA/N spleen |                  |                  |
|      |       |                 |              |                               |     |     |     |                  |                  |                  |
|      |       |                 |              |                               |     |     |     |                  |                  |                  |
| II   | CBA/H-T₆T₆ lymph node (5 × 10⁴) + PBS | 80 ± 8         | 214 ± 32     | 355 ± 54                      | 162 ± 10 | 136 ± 15 | 151 ± 12 |
|      |       |                 |              |                               |     |     |     |                  |                  |                  |

* Number of cells cultured.
† Mean number of colonies in three to four replicate cultures ± SD.

Colony Formation by Mixtures of CBA/N and Normal B Cells. To exclude active suppressor mechanisms in CBA/N lymphoid cell suspensions, mixtures of normal and CBA/N cells were cultured together (Table II). CBA/N cells did not interfere with, and even occasionally potentiated slightly, the formation of colonies by normal B cells. This slight enhancement was expected since addition of nonlymphoid cells to unpotentiated B-cell cultures increases cloning efficiency (1).

Mitogen Responses of CBA/N B Cells in Liquid Cultures. It has been documented that CBA/N B cells are capable of subnormal but detectable proliferative responses to certain mitogens in liquid cultures (8-10). Since mitogens native to agar are important for B-colony formation (2), it was important to test the ability of CBA/N mice to respond to these mitogens. Reactivity of CBA/N B cells to LPS, as assessed by ¹²⁵I-iododeoxyuridine (¹²⁵IUDR) uptake after 3 days in liquid culture, was variable but often quite vigorous (Table III). The proliferative responses of CBA/N B cells to agar-derived mitogens or dextran sulfate was more consistently lower than that of CBA/H or CBA/Cum lymphocytes but were still often measurable. When low responses to mitogens in liquid cultures were obtained, they could be restored by the addition of normal cells (Fig. 1). It has been reported that mitogen-stimulated lymphocytes can incorporate radioactive
TABLE III

Mitogen Responsiveness of CBA/N Lymphocytes in Liquid Cultures*

| Mitogen      | CBA/N spleen | CBA/N lymph node | CBA/Cum or CBA/H-T₂T₄ spleen | CBA/Cum or CBA/H-T₂T₄ lymph node |
|--------------|--------------|------------------|-----------------------------|----------------------------------|
| AM           | 3.5, 5.6, 1.9, 0.9 | 1.4, 1.0, 1.1 | 7.1, 10.7, 13.6 | 7.0, 3.1, 9.7 |
| LPS          | 15.2, 28.1, 14.8, 2.6 | 1.2, 1.0, 1.5 | 5.7, 6.7, 13.0 | 4.1, 2.9, 3.3 |
| DS           | 0.7, 1.0, 1.1, 0.5 | 2.0, 1.2 | 2.0, 0.6, 0.5 | 2.0, 0.5, 1.1 |
| Con A        | 0.3, 1.7, 1.3, 0.6 | 0.6, 1.0, 1.1 | 0.1, 0.1, 0.3 | 0.4, 0.4, 0.7 |
| +FCS         | 3.7, 6.6, 4.2, 3.7 | 3.7, 1.2 | 1.2, 3.2, 2.0 | 8.6, 11.1, 5.0 |
|             | 8.7, 90.5, 54.3, 33.6 | 55.7, 7.9 | 1.7, 3.0, 3.1 | 5.3, 15.6, 5.3 |
|             | 2.0, 1.6, 2.1, 1.2 | 0.7, 0.5 | 3.3, 2.7, 3.4 | 2.0, 1.1, 1.5 |
|             | 42.6, 206.9, 198.4, 90.5 | 5.4, 4.5, 17.8 | 5.4, 4.5, 17.8 | 1.1, 2.3, 13.6 |

*Data are means of triplicate cultures expressed as the ratio of 125IUdR incorporation in mitogen-containing cultures to that of the same cells cultured in medium alone. Each column of figures is the result of separate experiments with unpooled cell suspensions from individual mice in cultures containing 5% fetal calf serum or serum-free medium.

To determine if CBA/N B cells lack the ability to proliferate over a 5 day period as is required for colony formation and to test the validity of using 125IUdR uptake as an index of cell division, in a single experiment hemocytometer cell counts were performed on 3- and 5-day liquid cultures (Table IV). Isotope incorporation agreed reasonably well with actual cell recoveries and the presence of large, presumably responding, cells in the cultures. These findings suggest that the failure of CBA/N B cells to form colonies in a mitogen-stimulated gel culture is not due to total unresponsiveness to the mitogens that are present.

**Differentiation of CBA/N B cells in Liquid Cultures.** In addition to activating B cells to proliferate, certain mitogens cause maturation to high-rate immunoglobulin-secreting plasma cells. Since CBA/N cells display some proliferative responsiveness to mitogens, their ability to differentiate during 3–5 days of liquid culture with LPS was tested. Using the same conditions as for the previously discussed proliferation assays, CBA/N B-cell cultures contained almost as high a percentage of well-developed plasma cells as the controls. Differentiation is known to be very dependent on cell density (19, 21), so cultures were prepared under low-density conditions which favor expression of all classes of immunoglobulin. Normal spleen cells matured well under these conditions but surprisingly CBA/N cells proliferated poorly and very few large IgM-containing cells were found. An experiment illustrating these points is shown in Table V. Other experiments utilizing a scaled up version of the protocol employed here confirmed that cell density was the single variable which deter-
Culture in Serum Containing Medium  
Culture in Serum-Free Medium

**Fig. 1.** Mitogen response of mixtures of CBA/N and normal lymph node cells in liquid culture. A constant number of cells mixed in different ratios were cultured with LPS (open triangles), agar mitogens (closed squares), DS (open circles), or PBS (closed circles) in either 5% fetal calf serum or in serum-free medium. Each point is the mean $^{3}H$Tdr incorporation of triplicate cultures after 3 days.

| Mitogen | Day 3 | Day 5 |
|---------|-------|-------|
| PBS     | % Recovery | % Large | $^{3}H$Tdr (cpm) | % Recovery | % Large | $^{3}H$Tdr (cpm) |
| CBA/N   | 10 | 22 | 88 | 21 | 11 | 119 |
| AM      | 9 | 13 | 540 | 24 | 14 | 737 |
| LPS     | 35 | 44 | 9,467 | 64 | 40 | 2,065 |
| DS      | 12 | 9 | 107 | 3 | 1 | 61 |
| Con A   | 85 | 75 | 89,752 | 269 | 89 | 8,465 |

| Mitogen | Day 3 | Day 5 |
|---------|-------|-------|
| PBS     | % Recovery | % Large | $^{3}H$Tdr (cpm) | % Recovery | % Large | $^{3}H$Tdr (cpm) |
| CBA/H-T6 | 42 | 21 | 1,796 | 22 | 28 | 707 |
| AM      | 41 | 68 | 5,982 | 63 | 44 | 1,696 |
| LPS     | 104 | 79 | 14,659 | 110 | 75 | 3,330 |
| DS      | 21 | 68 | 6,618 | 32 | 72 | 7,534 |
| Con A   | 143 | 81 | 59,873 | 231 | 86 | 7,374 |

TABLE IV  
Proliferative Ability of CBA/N B Cells in Mitogen-Stimulated Liquid Cultures Assessed by Cell Counting and $^{3}H$Tdr Uptake

| Mitogen | Day 3 | Day 5 |
|---------|-------|-------|
| PBS     | % Recovery | % Large | $^{3}H$Tdr (cpm) | % Recovery | % Large | $^{3}H$Tdr (cpm) |
| CBA/N   | 10 | 22 | 88 | 21 | 11 | 119 |
| AM      | 9 | 13 | 540 | 24 | 14 | 737 |
| LPS     | 35 | 44 | 9,467 | 64 | 40 | 2,065 |
| DS      | 12 | 9 | 107 | 3 | 1 | 61 |
| Con A   | 85 | 75 | 89,752 | 269 | 89 | 8,465 |

Liquid microcultures were harvested, pooled, and the percentage of the starting number of viable cells recovered and the percentage of large cells determined by phase-contrast microscopy. Identical cultures were pulsed with $^{3}H$Tdr, and the amount of TCA precipitable radioactivity determined 4 h later. All cultures contained 5% fetal calf serum.

mined normal or defective activation and maturation of CBA/N B cells in liquid cultures (data not shown). Furthermore, many of the IgM-containing cells observed in low-density CBA/N liquid cultures were found in clumps, and aggregation in the cultures could be responsible for the small number of these cells which were seen.

**Serum Inhibitor Levels in CBA/N Mice.** Normal mouse serum is known to contain inhibitors for various in vitro assays (22, 23), and the B-cell-cloning assay is no exception. Addition of undiluted serum from several different strains of mice resulted in greater survival of single, small cells, but almost completely inhibited colony formation. Serum samples from six different CBA/Cum and five different CBA/N mice were titered for this inhibitor activity. Using the maximum final dilution of serum necessary to inhibit proliferating foci by 50%
The Effect of Cell Density on CBA/N B-Cell Proliferation and Differentiation

| Culture conditions | Cells | LPS | % Recovery | % IgM* cells | 
|--------------------|------|-----|------------|--------------|
|                    |      | 3 days | 5 days | 3 days | 5 days | 3 days | 5 days | 3 days | 5 days | 3 days | 5 days | 3 days | 5 days |
| Conditions used throughout this study to assess mitogen stimulation (4 × 10³ cells/culture, 7% CO₂, 5% FCS) | CBA/N | 5 | 106 | 75 | 25.9 | 22.2 | 12,689 | 5,063 |
| | 50 | 86 | 107 | 27.3 | 35.9 | 10,845 | 3,833 |
| | CBA/H | 5 | 53 | 20 | 4.5 | 14.4 | 413 | 338 |
| | 50 | 162 | 108 | 37.6 | 38.0 | 6,038 | 559 |
| Same as above except 5 × 10⁴ cells/culture | CBA/N | 5 | 5 | 2 | 0.1 | 0.1 | 20 | 32 |
| | 50 | 7 | 2 | 17.5 | 3.3 | 63 | 43 |
| | CBA/H | 5 | 10 | 3 | 2.4 | 6.6 | 23 | 22 |
| | 50 | 23 | 21 | 54.3 | 69.2 | 5,387 | 1,631 |
| Conditions used by Kearney and Lawton (19) (5 × 10³ cells/culture, 5% CO₂, 20% FCS) | CBA/N | 5 | 2 | 4 | 0.1 | <0.1 | 28 | 53 |
| | 50 | 9 | 1 | 5.8 | ND | 40 | 54 |
| | CBA/H | 5 | 13 | 69 | 40.9 | 63.6 | 7,896 | 9,819 |
| | 50 | 33 | 113 | 45.3 | 65.1 | 8,295 | 9,458 |

Cultures were initiated with cell suspensions from individual mice in Falcon Microtest II plates. The fraction of the starting number of viable cells recovered after 3 or 5 days of culture was determined by phase-contrast microscopy. All 500 intact cells were evaluated in cytocentrifuge preparations by immunofluorescence and cells with abundant, intensely fluorescent cytoplasm were recorded as IgM*.

as an index of inhibitor concentration, sample-to-sample variability was seen (range 1/30 to 1/90), but no difference was attributable to the strain of origin of the serum (data not shown). These experiments indicate that CBA/N mice have adequate, but not elevated, levels of serum factors which may be important for B-cell regulation in vivo.

Incidence of Other Functional Hemopoietic Cells in CBA/N Mice. Further evidence for restriction of the lesion of CBA/N mice to B cells was obtained by determining the frequency of CFU-c and CFU-s in the marrow of CBA/N mice. These values were within normal range (79 ± 11 CFU-c and 22 ± 2 CFU-s/5 × 10⁴ CBA/N bone marrow cells; 73 ± 7 CFU-c and 16 ± 4 CFU-s/5 × 10⁴ CBA/H-T₆T₆ bone marrow cells).

B-Cell Colony Formation by C3H Mice. We had previously noted that lymphocytes from C3H/HeJ mice were normal in their ability to form B-cell colonies (2). This was based on several experiments employing mice from a colony of C3H/HeJ mice on which we maintain the W⁺ mutation. Heterozygous W⁺/+ or wild-type +/+ C3H/HeJ mice had as many colony-forming B cells as C3H/St mice. More recently, we obtained other C3H/HeJ mice which were not associated with the W⁺ mutation colonies from The Jackson Laboratory, and colony formation by their B lymphocytes was defective (Table VI). Unlike cell suspensions from CBA/N mice, there were a small number of colony-forming cells in the C3H/HeJ spleen and lymph nodes which formed normal size colonies and whose size and number were slightly potentiated by agents that had no effect on CBA/N B cells.

Liquid Culture Mitogen Responses by C3H Mice. As has been often re-
**CBA/N AND C3H/HeJ B LYMPHOCYTES**

**Table VI**

*B Lymphocyte Colony Formation by Different Strains of C3H Mice*

| Experiment | Experiment | Experiment |
|------------|------------|------------|
|            | I          | II         | III        |
| C3H/HeJ    | 5 × 10⁴ Spleen cells + PBS | 4 ± 2* | 2 ± 1 | 2 ± 1 |
|            | LPS        | 11 ± 1     | 4 ± 2     | 2 ± 2 |
|            | SRBC       | 6 ± 2      |           |     |
|            | Macrophages| 21 ± 4     |           |     |
| 10⁶ Lymph node cells + PBS | 5 ± 2      | 2 ± 2     | 1 ± 1   |
|            | LPS        | 27 ± 5     | 17 ± 8   | 0    |
|            | SRBC       | 12 ± 9     |           |     |
|            | Macrophages| 25 ± 6     |           |     |
| C3HeB/FeJ  | 5 × 10⁴ Spleen cells + PBS | 197 ± 20 | 237 ± 14 | 137 ± 43 |
|            | LPS        | 303 ± 17   | 377 ± 33 | 174 ± 51 |
|            | SRBC       | 340 ± 17   |           |     |
|            | Macrophages| 286 ± 26   |           |     |
| 10⁶ Lymph node cells + PBS | 170 ± 10   | 185 ± 19  | 155 ± 17 |
|            | LPS        | 341 ± 30   | 298 ± 15 | 213 ± 13 |
|            | SRBC       | 338 ± 41   |           |     |
|            | Macrophages| 324 ± 32   |           |     |
| C3H/HeJW⁺/+ | 5 × 10⁴ Spleen cells‡ + PBS | 240 ± 17 |     |     |
|            | LPS        | 241 ± 18   |     |     |
| 10⁶ Lymph node cells + PBS | 55 ± 13    |     |     |     |
|            | LPS        | 32 ± 9     |     |     |

* Mean number of colonies in three to four replicate cultures ± SD.
‡ Similar results were obtained with cells from homozygous +/+ mice from this colony.

Ported, C3H/HeJ mice were poor responders to LPS in liquid cultures (Table VII). Excellent stimulation was seen, however, when the same cell suspensions were incubated with agar mitogens or dextran sulfate. Not surprisingly, our colony of C3H/HeJ W⁺/+ mice responded to LPS in keeping with their colony-forming ability.

**Potentiation of B-Cell Cloning by CBA/N and C3H/HeJ Macrophages.** Formal proof that accessory cells which regulate B-cell clonal proliferation are intact in these B-cell defective mice was obtained by using thioglycolate-activated peritoneal macrophages from them to potentiate normal CBA/Cum spleen or lymph node B cells. As shown in Table VIII, all strains of mice were equivalent in the ability of their macrophages to elaborate diffusable enhancing factors.

**Discussion**

The studies presented here provide evidence that functional B cells capable of clonal proliferation in soft agar culture are completely lacking in CBA/N mice and confirm that this is an intrinsic B-cell defect. Manipulation of the culture conditions, such that colony number is a linear function of the number of functional B cells present, did not facilitate colony formation by up to 10⁶ CBA/N
Table VII

Responses of C3H Mice to B-Cell Mitogens in Liquid Culture

|                     | Experiment I |          |          | Experiment II |          |          |
|---------------------|--------------|----------|----------|--------------|----------|----------|
|                     | -FCS         | +FCS     |          | -FCS         | +FCS     |          |
| C3H/HeJ             | Medium       | 693      | 1,241    | 382          | 444      |          |
| AM                  | 7,287 (10.7) | 21,208 (17.1) | 8,925 (23) | 10,379 (23) |          |          |
| LPS                 | 2,015 (2.9)  | 2,261 (1.8) | 596 (2)  | 696 (2)      |          |          |
| DS                  | 2,331 (3.4)  | 10,842 (8.7) | 4,771 (12) | 6,903 (16)  |          |          |
| C3HeB/FeJ           | Medium       | 1,055    | 858      | 945          | 1,617    |          |
| AM                  | 4,961 (4.7)  | 9,340 (10.9) | 14,886 (16) | 14,305 (9) |          |          |
| LPS                 | 8,004 (7.6)  | 14,185 (16.5) | 13,736 (15) | 12,219 (8) |          |          |
| DS                  | 2,702 (2.6)  | 8,553 (10.0) | 8,847 (9)  | 15,728 (10) |          |          |
| C3H/HeJW+/+         | Medium       | 1,151    | 884      | 901          | 1,667    |          |
| AM                  | 3,148 (2.7)  | 4,951 (5.6) | 10,686 (16) | 14,305 (9) |          |          |
| LPS                 | 5,485 (4.8)  | 11,620 (13.1) | 13,736 (15) | 12,219 (8) |          |          |
| DS                  | 5,207 (4.5)  | 10,789 (12.2) | 8,847 (9)  | 15,728 (10) |          |          |

Data represent the mean ³HThd uptake (cpm) of triplicate cultures (stimulation over background) prepared in 5% fetal calf serum or serum-free medium.

Table VIII

Ability of Macrophages from Different Strains of Mice to Enhance B-Lymphocyte Colony Formation

| Cells cultured                  | Potentiated by: | Colonies/culture ± SD |
|---------------------------------|-----------------|------------------------|
| CBA/Cum spleen (10⁴)            | PBS             | 28 ± 6                 |
|                                 | LPS             | 99 ± 11                |
|                                 | CBA/Cum Mφ      | 120 ± 12               |
|                                 | CBA/N Mφ        | 112 ± 13               |
|                                 | C3HeB/FeJ Mφ    | 127 ± 38               |
|                                 | C3H/HeJ Mφ      | 103 ± 26               |
|                                 | B6D2F/·Cum Mφ   | 98 ± 25                |
| CBA/Cum lymph node (5 x 10⁴)    | PBS             | 86 ± 9                 |
|                                 | LPS             | 263 ± 27               |
|                                 | CBA/Cum Mφ      | 276 ± 27               |
|                                 | CBA/N Mφ        | 267 ± 22               |
|                                 | C3HeB/FeJ Mφ    | 277 ± 35               |
|                                 | C3H/HeJ Mφ      | 304 ± 51               |
|                                 | B6D2F/·Cum Mφ   | 287 ± 41               |

Thioglycolate-activated peritoneal macrophages (Mφ) were allowed to adhere to culture dishes, washed, and covered by a spacer gel as described in the Materials and Methods. Cultures of spleen or lymph node cells were prepared over these and resulting colonies were scored 5 days later.

cells in single culture dishes. Numbers and function of bone marrow CFU-c and CFU-s, functional peritoneal macrophages, and levels of serum inhibitors were all normal in CBA/N mice. As in previous studies (8), cell mixing experiments negated the possibility that active suppressor mechanisms could be responsible for the CBA/N deficiency.
Some proliferative responsiveness to agar-derived mitogens was suggested by 3-day liquid cultures of CBA/N cells and LPS responses in such cultures were often surprisingly large. Nonspecific B-cell activation has been achieved with liquid cultures of CBA/N B cells with high concentrations of LPS (8) and serum IgM levels of CBA/N mice are elevated after LPS injection (11). CBA/N B cells differentiated to plasma cells containing large amounts of immunoglobulin in cultures prepared at high initial cell density with LPS. The same cells proliferated and differentiated poorly compared to normal B cells, however, when the number of cells per culture was decreased eightfold. These findings suggest that defective mice may have B cells that are intrinsically unable to divide and mature in dilute in vitro culture or form colonies in semisolid culture where cell contact is minimized. The cell density in the low-density liquid cultures was approximately $1.8 \times 10^5/\text{mm}^2$ after settling to the bottom of the dishes as compared to a maximum of $1 \times 10^6/\text{mm}^2$ in the gel cultures. Alternatively, these particular culture conditions may select for a subpopulation of B cells which CBA/N mice lack. Cell density has been reported by others to have profound effects on the efficiency of in vitro B-cell differentiation and the classes of immunoglobulin which are expressed (19, 21).

CBA/N mice possess B cells which are capable of responding to antigens via participation of T cells, although the magnitude of these responses are often slightly subnormal (8, 9), and the quality of the antibody produced may differ from that produced by normal animals (24). The most striking abnormality of these mice is their inability to respond to those antigens that elicit B-cell responses in the absence of functional T cells. The total failure of CBA/N B cells to form in vitro colonies suggests that the assay preferentially, and perhaps exclusively, detects a subpopulation of B cells whose function is not dependent on T cells.

Several investigators have suggested the existence of a discrete functional category of thymus-independent B cells (termed B1) which might precede in ontogeny and/or phylogeny, a distinct thymus-dependent B-cell population (B2) (25, 26). The reverse association, namely, that B2 cells might be an immature form of B1, has also been suggested (27, 28), and CBA/N mice are said to have an immature distribution of B cells with respect to cell-surface immunoglobulin (13). A possibility that is still open is that these two categories of B cells represent completely separate avenues of differentiation, each one of which contain cells of different maturational stages.

Evidence has been presented that in certain conditions of antigen priming, B1 cells have an apparent size difference from B2 cells (29), and that responses to most, if not all, thymus-independent antigens are mediated by complement receptor-negative (CR-) B cells without participation of complement components (30-35). The most immature Ig$^+$ B cells lack CR; some of these may eventually become Ig$^+$, CR$^+$, and this maturation is reported to be genetically controlled (36-38). The incidence of CR$^+$ lymphoid cells in adult tissues is variable (39), but Ig$^+$, CR$^-$ lymphocytes usually comprise 30-60% of splenic B cells (34, 38). Interestingly, cells taken from in vitro colonies at the end of culture have been found to lack CR (40). Improvements in methodology have increased the efficiency of B-cell cloning, but still only a minority of B cells routinely form colonies. CBA/N mice are slightly deficient in splenic Ig$^+$ cells.
Further analysis of the CR status of clonable B cells at the initiation of culture, and the possible role of activated complement in colony formation should test the relatedness of these findings. That is, the cells that are missing in CBA/N lymphoid tissues and that form colonies in vitro might be Ig⁺, CR⁻ B1 type lymphocytes.

A number of observations suggest that although B1 cells and colony-forming cells may be distinct from B2 cells, neither is homogeneous. It has been shown that B1 cells include virgin and antigen-experienced cells of different sizes (29, 41). Differential responsiveness of various populations of B cells to different T-independent mitogens/polyclonal activators with production of different classes of Ig is well-known (references 42-44; P. W. Kincade and J. F. Kearney, unpublished observations). Most colony-forming B cells are probably IgM⁺ and Ia⁺, but since we have only examined cells from mature lymphoid organs under unpotentiated conditions, the existence of a minority of functional cells lacking one or both of these surface receptors is possible (3). B cells capable of colony formation are present among the earliest functional B cells in fetal liver and have been found in every lymphoid tissue of adult animals with the exception of the thymus (1, 45). Preliminary experiments with a rabbit antiserum to putative mouse δ (46) indicate that 2/3 to 3/4 of colony-forming B cells are δ⁺ and hypersensitive to the antiserum, whereas the remainder is refractory to treatment with it (P. W. Kincade and R. M. E. Parkhouse, unpublished observations). All of these observations are consistent with the notion that B1 and B2 are separate categories of cells which may both include diverse subcategories of cells. The possible lineal relationship of the two, as well as the origin, average lifespan, and migration patterns of B1 cells, can be investigated by injecting various populations of normal cells into CBA/N mice and following their fate with the in vitro cloning technique.

Liquid and semisolid culture responses of C3H/HeJ B cells form an interesting contrast to those of CBA/N mice. C3H/HeJ mice taken from an appropriate breeding colony have approximately 10% as many colony-forming B cells as other histocompatible C3H substrains. Previously, the only known deficit associated with these mice was in reactivity to certain forms of endotoxin (LPS) (14-18). Paradoxically, they mounted excellent liquid culture proliferative responses with agar-derived mitogens which are important for colony formation. The results are, therefore, subject to several different interpretations. A trivial one is that LPS is a vital factor in colony formation. The most likely source of LPS contamination is the agar used to support colonies since the mitogens native to our fetal calf serum differ from LPS in requiring 2 ME for B-cell activation (P. W. Kincade, unpublished observations). The importance of contaminating LPS in colony formation can be tested by adding anti-LPS antibodies to the cultures, since free antigen-antibody complexes are not inhibitory (3). A second possibility is that 3-day liquid culture responses do not adequately assess the ability of B cells to undergo the prolonged division at infinitely low-cell density necessary for colony formation. These possibilities are under investigation. It is also tenable that C3H/HeJ mice have a quantitative deficit in B1. It has been reported that they are capable of in vitro and in vivo responses to thymus-independent antigens other than LPS (15-17), but it is necessary to test their functional capability in a system where responding cells are limiting (41). The
incidence of cells with various B markers and density of B-cell surface Ig are reportedly normal in C3H/HeJ mice (16, 47).

From numerous parallel studies of the requirements for liquid culture responsiveness and colony formation (P. W. Kincade, unpublished observations), it is apparent that proliferative ability is a necessary, but not sufficient, requirement for colony formation. The latter process is much more complex and is dependent on 2 ME, macrophage-derived factors, and substances in fetal calf serum in addition to an appropriate stimulating mitogen. The selectivity of the technique, which is demonstrated by these studies, suggests that it will be invaluable in defining functional B-cell subpopulations, their requirements for activation and differentiation, and their status in immune-deficiency diseases.

Summary

B cells from CBA/N mice did not form colonies in semisolid agar cultures under circumstances where normal B-cell clonal proliferation is linear with respect to the number of functional cells cultured. This was not due to the unresponsiveness of CBA/N cells to mitogens, and under appropriate liquid culture conditions many CBA/N lymphocytes differentiated to plasma cells containing large amounts of IgM in response to LPS. On the other hand, the same cells proliferated and matured poorly in liquid cultures prepared at low-cell density. The frequency of granulocyte-macrophage progenitors and multipotential hemopoietic stem cells in bone marrow, ability of peritoneal macrophages to elaborate soluble enhancing factors, and levels of serum inhibitors were normal in CBA/N mice. Together with the results of cell-mixing experiments, these findings confirm the selective and intrinsic nature of the CBA/N deficiency. It is suggested that the B-cell-cloning technique may be of value in selectively enumerating and assessing functional capability of thymus-independent B cells. C3H/HeJ mice which have previously only been known to be hyporesponsive to certain forms of lipopolysaccharide had a subnormal incidence of colony-forming B cells.

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