

B-CELL DIFFERENTIATION IN THE CBA/N MOUSE

I. Slower Maturation of Mitogen and Antigen-Responsive B Cells in Mice Expressing an X-linked Defect*

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The CBA/N strain of mouse, derived in 1966 from the CBA/H strain (1), carries an X-linked mutation whose expression affects a number of B-cell functions. The B cells in adult CBA/N mice have many of the characteristics of those found in neonatal mice, including a higher density of surface IgM and a lower expression of B-cell differentiation markers, such as Ia, Mls, Lyb3, and Lyb5.1 (2-5). In addition, there is a change in the pattern of responsiveness of B cells in these mice to various groups of antigens and mitogens. B-cell polyclonal mitogens (lipopolysaccharide [LPS])¹, thymus-dependent antigens (sheep erythrocytes, [SRBC]), and one group of thymus-independent antigens termed TI-1 (trinitrophenyl [TNP]-LPS and TNP-*Brucella abortus*) (6) all stimulate responses in these mice (1, 2, 6-12). In contrast, this strain apparently has a complete lack of responsiveness to a second group of thymus-independent antigens, TI-2 (TNP-Ficoll, Poly I:C, SSS-III) (9, 10, 13-15) which appear to stimulate a more mature population of B cells (6). The possibility exists that there is a relationship between the pattern of surface-marker expression and the pattern of responsiveness to various antigens and mitogens.

The purpose of this work is to examine the effect of age on the B-cell response capabilities of CBA/N mice. Although spleen cells from these defective mice respond in vitro to both mitogens and thymus-dependent antigens, the levels of responsiveness reported have varied greatly (1, 2, 6, 10, 14, 16). The experiments described in this paper reveal that responses of CBA/N and (CBA/N × DBA/2)F₁ male mice to the mitogen, LPS, and the thymus-dependent antigen, SRBC, increase markedly with age. In addition, spleen cells from these mice show a distinct age-related increase in the percentage of surface Ig⁺ cells. These findings show that the X-linked mutation in the CBA/N mouse affects maturation rates of many B-cell functions during a critical time range, which may account for the variability seen in experiments in the reported literature.

Materials and Methods

Mice. CBA/N and (CBA/N × DBA/2)F₁ breeding colonies were established using CBA/

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¹ *Abbreviations used in this paper:* BSS, Hanks' balanced salt solution; FCS, fetal calf serum; LPS, lipopolysaccharide; SRBC, sheep erythrocytes; [³H]TdR, [³H]thymidine, TNP, trinitrophenyl; TNP-BA, TNP-conjugated *Brucella abortus*.

N mice provided by Dr. Priscilla Campbell, National Jewish Hospital, Denver, Colo., and DBA/2 male mice purchased from The Jackson Laboratory, Bar Harbor, Maine.

Lipopolysaccharide. Hot phenol-extracted K235 LPS, prepared as described elsewhere (17), was the gift of Kathleen Kelly, University of California, Irvine, Calif. LPS was used at a concentration of 1 $\mu\text{g}/\text{ml}$ unless otherwise indicated.

LPS Mitogenic and Polyclonal Responses. Spleen cells were cultured in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 U penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin (18). Cells were cultured in 0.2-ml volumes in Microtest II plates (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) at a density of 2.5×10^6 nucleated cells/ml. Cultures were maintained at 37°C in an atmosphere of 7% O₂, 10% CO₂, and 83% N₂ until the day of assay.

For measurement of LPS mitogenic activity, cells were assayed on day 2 or 3 for their ability to incorporate [³H]thymidine ([³H]TdR). Each culture was pulsed for 6 h with 0.25 μCi of 8 Ci/mM [³H]TdR (ICN, Irvine, Calif.). Cells were harvested onto glass fiber filter strips (Whatman Inc., Clifton, N. J.) using an automated MASH II cell harvester (Microbiological Associates, Walkersville, Md.). Filters were dried, then counted using a toluene/terphenyl liquid scintillation fluid (19).

LPS polyclonal responses were measured by enumerating the number of TNP-SRBC plaque-forming cells per culture using the Cunningham modification of the hemolytic plaque assay (20). SRBC, (Colorado Serum Co., Denver, Colo.), were conjugated to TNP as previously described (21).

Limiting Dilution Assay. Principles and techniques involved in the use of the limiting dilution assay have been described in detail elsewhere (22). Unless indicated otherwise, the culture system used in these assays consists of Iscove media (23) supplemented with 5% FCS, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 U of penicillin, and 50 $\mu\text{g}/\text{ml}$ of streptomycin. To insure that spleen cultures were limiting only for the responding B-cell precursors, 3×10^4 filler cells were added to each 10- μl culture. Antibody production was tested on day 4 (LPS) or 6 (SRBC) using the automated spot test designed by Lefkovits (24, 25).

Filler Cells. Two types of filler cells were used for the limiting dilution assays. For responses to SRBC, 2-5-mo-old female (CBA/N \times DBA/?)F₁ mice were low-dose primed by intravenous injection of 2×10^6 SRBC. 5-7 d later, spleens were removed and single cell suspensions were made in Hanks' balanced salt solution (BSS) (18) at a density of 1×10^8 cells/ml. Cells were irradiated (1,200 R) using a Maximar R 100 x-ray machine (General Electric Co., Los Angeles, Calif.).

For responses to LPS, thymus cells from (CBA/N \times DBA/2)F₁ male and female mice were pooled and used without further preparation.

Fluorescence Microscopy. Rabbit anti-mouse Ig was provided by Dr. Robert Coffman, Stanford University, Palo Alto, Calif., and was prepared as previously described (26). This antisera was absorbed on BALB/c thymus cells and diluted 1:20 in BSS supplemented with 0.05% NaN₃. Fluorescent sheep anti-rabbit Ig was purchased from the Pasteur Institute, Paris, France, and diluted 1:100 before use. Both antisera were deaggregated by spinning at 100,000 g for 1 h.

For determination of the percentage of surface Ig⁺ (sIg⁺) spleen cells, 5×10^6 cells, washed in BSS-NaN₃, were resuspended in 0.1 ml of the 1:20 dilution of rabbit anti-mouse Ig, then incubated at 4°C for 30 min. After two washes in BSS-NaN₃, this procedure was repeated for the fluorescent sheep anti-rabbit Ig. Fluorescent cells were enumerated with the use of a Leitz Dialux microscope (E. Leitz, Inc., Rockleigh, N. J.) with a Ploem fluorescent attachment.

Results

The Effect of Age on LPS Mitogenic Responses of CBA/N and (CBA/N \times DBA/2)F₁ Mice. The (CBA/N \times DBA/2)F₁ is often used in the study of the CBA/N defect, as males express the X-linked defect whereas females respond normally in most assays. This combination provides phenotypically defective and normal mice which are identical both in age and conditions under which they are raised. In our preliminary experiments comparing the in vitro responses of spleen cells for male and female F₁

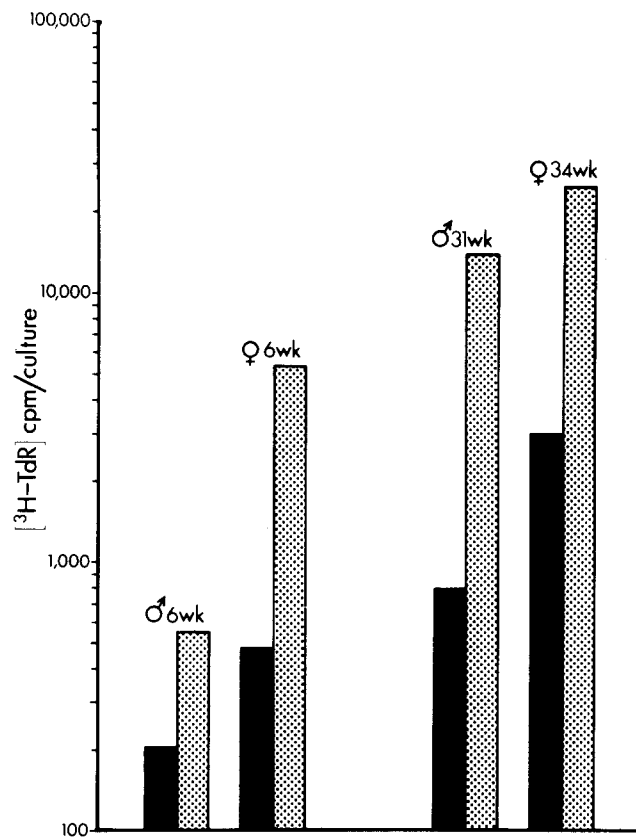


FIG. 1. Comparison of LPS mitogenic responses of young and old (CBA/N \times DBA/2) F_1 mice. Young (6 wk) and old (31 and 34 wk) male and female F_1 spleen cells were cultured at 5×10^6 cells per culture and were assayed on day 2 for incorporation of [³H]thymidine. Data presented are an average of quadruplicate cultures. ■, control; ▨, LPS.

mice to the mitogen LPS, considerable variability was seen in the male F_1 response which was not observed in the female response. Examination of the data indicated that the ability of male mice to respond to LPS may vary with age.

To test this possibility, LPS mitogenic responses of 6- and 30-wk-old male spleen cells were compared to those of 6- and 34-wk-old female spleen cells. Mitogenic responses to LPS were measured by determining the incorporation of [³H]thymidine during a 6-h pulse on day 2 of culture, and the results are shown in Fig. 1. When the responses of the 6-wk-old F_1 littermates were compared, the F_1 male spleens were found to respond poorly to LPS (550 cpm/culture), with F_1 female spleens showing responses which were 10-fold higher (5,500 cpm/culture). At 30 wk, the response of the F_1 male spleen cells to LPS had increased to a level which approached that observed for F_1 female spleen cells of a similar age (Fig. 1).

To determine the rate at which the mitogenic responsiveness to LPS increases in mice expressing the X-linked defect, spleen cells from CBA/N mice between 1 and 52 wk of age were tested. Fig. 2 contains data from the LPS responses of 3, 9, 13, 17, and 52-wk-old mice. Mitogenic responses to LPS in spleen cell cultures of these mice increased steadily during the period of 3–17 wk, then this increase slowed. As a result,

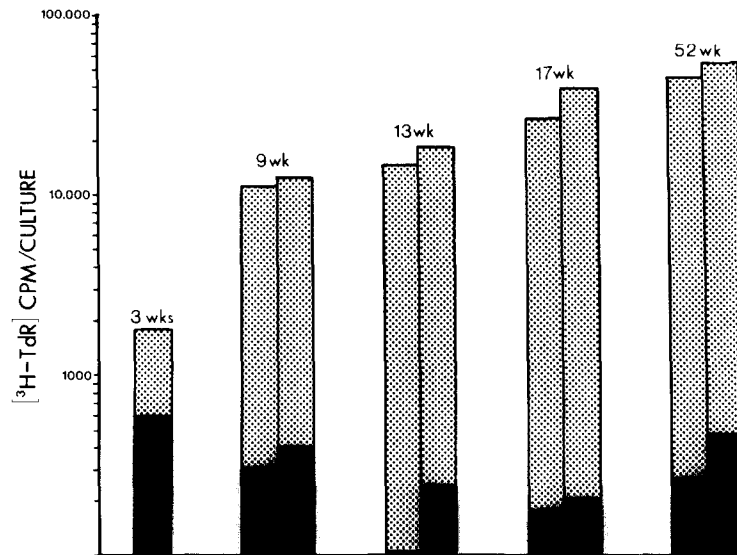


FIG. 2. Age-related increases in LPS mitogenic responses of CBA/N mice. Duplicate 3, 9, 13, 17, and 52-wk-old mice were assayed on day 2 for mitogenic responses to LPS. Duplicate spleens of 3-wk-old mice were combined due to low number of cells. Data presented are an average of quadruplicate cultures. ■, control; ▨, LPS.

little change was seen between the responses at 17 and 52 wk of age, which were at a level that approximates those observed in other mouse strains. This slower acquisition of LPS responsiveness, therefore, occurs in both strains of mice (CBA/N and F₁ male) expressing the X-linked defect and, more importantly, occurs during a time period which is critical for most immunological assays. Mice in the age range of 8–12 wk are widely used as a source of cells for mitogenic and immune response studies. The changing level of responsiveness during this period in CBA/N mice may account for the variability described in the reported literature.

The Effects of Cell Density and LPS Concentration on the LPS Mitogenic Responses of 7- and 13-wk-old F₁ Mice. The differing abilities of young and old F₁ male and CBA/N mice to respond to LPS could be the result of the culture conditions employed. To examine this, mitogenic responses of spleen cells from 7- to 13-wk-old F₁ mice were compared at various culture densities (10^5 – 10^6 cells/0.2 ml culture) and over a wide range of LPS concentrations (0.001–50 μ g/ml). Spleen cells from 7-wk-old F₁ male mice gave a low but measurable mitogenic response to LPS, which was considerably lower than the response of the F₁ female at each cell density and LPS concentration (Fig. 3A–C).

The mitogenic responses of spleen cells from 13-wk-old F₁ mice shown in Fig. 3 (panels D–F), are higher than those at 7 wk of age, but under no culture condition are the male responses equivalent to those of the age-matched F₁ female spleen cells. It is noticeable that for each cell density, the LPS dose-response profiles of male and female spleen cells are approximately similar. This suggests that the lower response of male spleen cells is a result of a lower number of LPS reactive precursors.

One feature of the above experiments requires emphasis. Consistent with other published data (2, 9, 10, 12, and 16) the background incorporation of radioactive thymidine in spleen cultures from mice expressing the X-linked defect are consistently

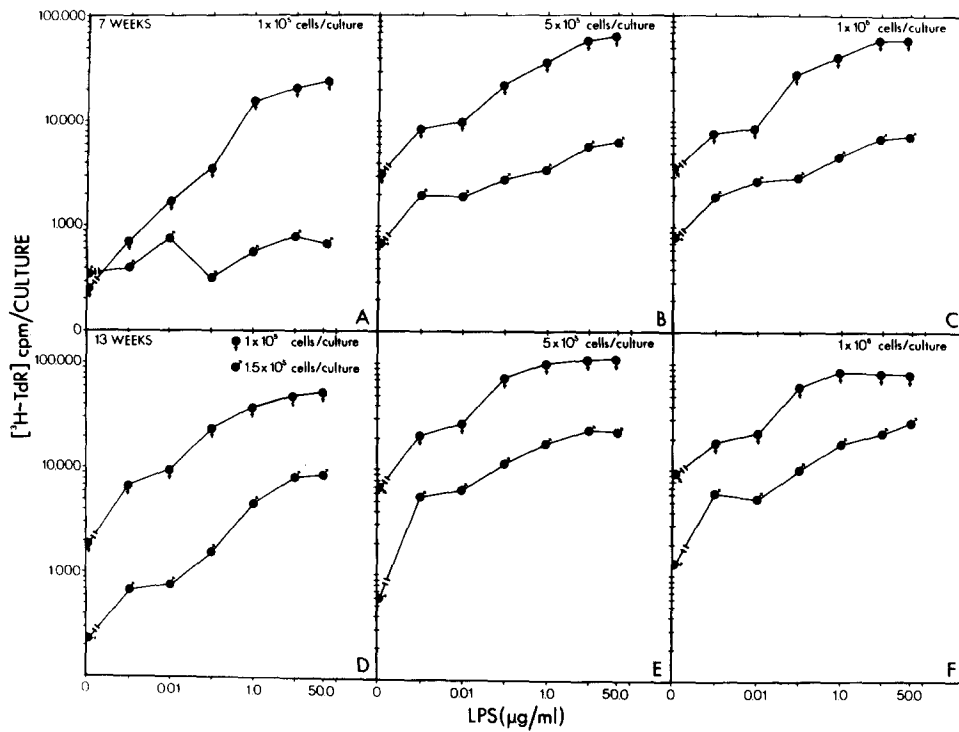


FIG. 3. Effect of cell density and LPS concentration on mitogenic responses of (CBA/N \times DBA/2) F_1 male and female spleen cells. 7- and 13-wk-old F_1 mice were tested for mitogenic responses on day 2. 7-wk-old male and female spleen cells (panels A-C) were cultured at 1, 5, and 10×10^6 cells/0.2 ml in the presence of various concentrations of LPS. For 13-wk-old mice (panels D-F) the male spleen cells were cultured at 1.5, 5, and 10×10^6 per culture, and female cells were cultured at the same cell densities used for 7-wk-old mice. Data presented are an average incorporation of quadruplicate cultures.

3- to 10-fold lower than those observed in spleen cultures of normal mice. The backgrounds remain low even though responsiveness to LPS is increasing dramatically. This may have significance as to the cellular site of the defect which is discussed below.

B-Cell Precursor Frequencies in Age-matched Male and Female F_1 Mice. The data presented above suggest the lowered mitogenic responses of F_1 male and CBA/N spleen cells are a result of a lower frequency of LPS-reactive precursors. If B-cell precursor frequencies do differ in the spleens of defective and normal mice, determination of these frequencies would provide an accurate method for comparing the response capabilities of these mice to a variety of antigens and mitogens. To investigate this point further, the frequency of B cells responsive to LPS and the thymus-dependent antigen, SRBC, were measured in the spleens of F_1 mice by the limiting dilution assay developed by Lefkovits (24). In this assay, responding spleen cells are cultured at limiting dilution in the presence of a constant number of irradiated spleen or thymus filler cells. To assay for responding cultures, the culture supernates are tested on day 4 (LPS) or 6 (SRBC) for the presence of antibody which will bind to SRBC. The precursor frequency can be determined directly by plotting the fraction of nonresponding cultures versus the number of responding spleen cells per culture. By

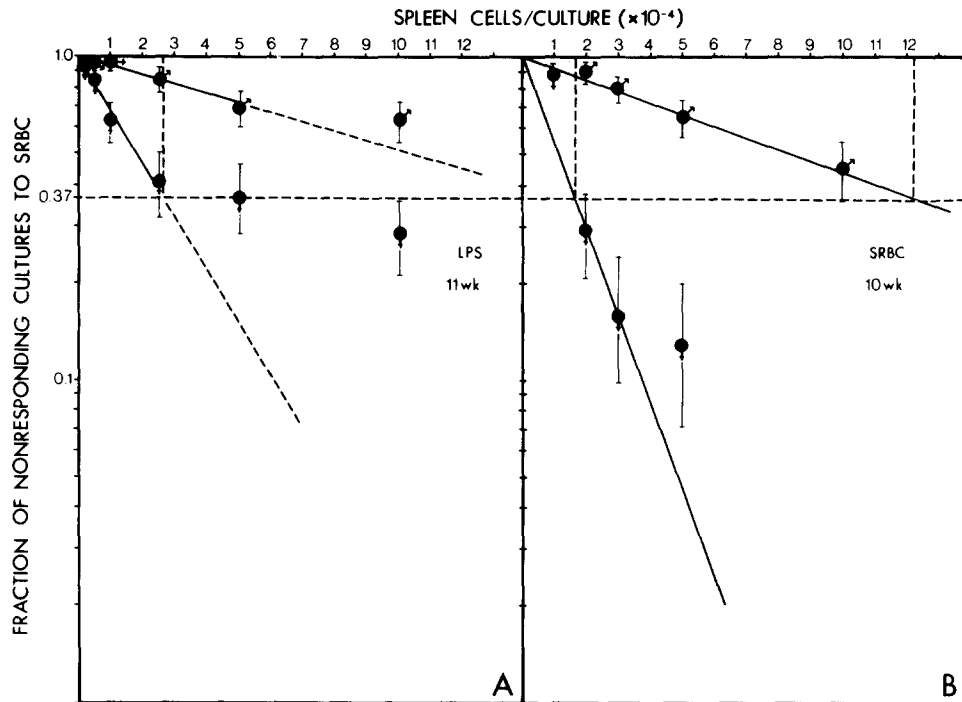


FIG. 4. LPS and SRBC-reactive B-cell precursor frequencies of male and female F_1 mice. Two sets of F_1 male and female littermates were assayed for the frequency of B cells forming antibody to SRBC after stimulation with LPS (A) or SRBC (B). Media for limiting dilution analysis of LPS-reactive SRBC precursors was RPMI-1640 supplemented as described for Iscove media in Materials and Methods. Culture supernates were assayed on day 4 (LPS) or 6 (SRBC) by an automated spot test for antibody which binds SRBC. Vertical bars represent 95% confidence levels. Precursor frequencies: (A) LPS, ♀ = 3.7×10^{-5} , ♂ = 7.6×10^{-6} ; (B) SRBC, ♀ = 6.2×10^{-5} , ♂ = 8.2×10^{-6} .

Poisson analysis, when 37% of the cultures are nonresponding, there is an average of one reactive precursor per culture (22).

Fig. 4 presents data for the limiting dilution analyses of LPS- and SRBC-responsive B cells in the spleens of two sets of F_1 male and female littermates. In both assays, the frequencies of precursors in the spleens of male F_1 mice were markedly lower than those of the female F_1 spleen cells. At 11 wk, the frequency of precursors in the F_1 spleens reactive to LPS differed fivefold. The male spleen contained few reactive precursors with a frequency of only 7.6×10^{-6} , whereas the female spleen contained much higher numbers with a frequency of 3.7×10^{-5} . A similar result was found for the SRBC precursor frequencies. In 10-wk-old F_1 mice, the male mouse gave approximately eightfold lower frequencies to SRBC with frequencies of 8.2×10^{-6} and 6.2×10^{-5} for the male and female mice, respectively. The curves in both assays are approximately linear, which indicates that our culture system is limiting only for the B-cell precursors. This lends further support to the earlier suggestion that the lower responses of male F_1 and CBA/N mice to LPS are a result of lower number of LPS responsive B-cell precursors in the spleen cell population.

In this and the following analyses of SRBC precursor frequencies, irradiated F_1 female spleen cells were used as fillers so that the culture system be limiting only for

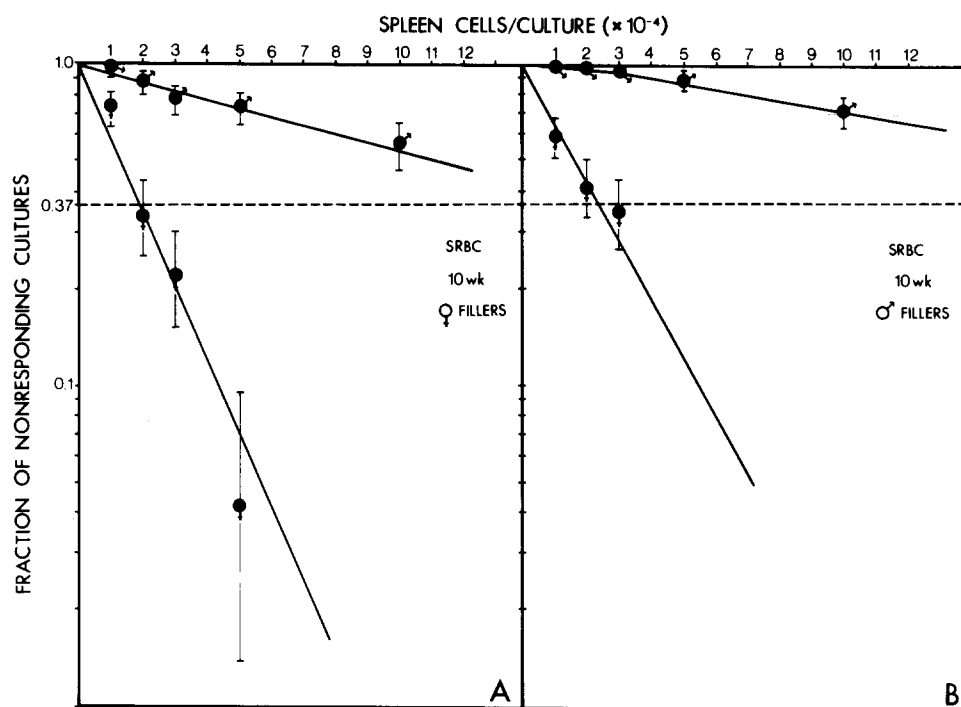


FIG. 5. Comparison of SRBC-reactive precursor frequencies in the presence of irradiated male and female filler cells. 9-wk-old male and female mice were primed to SRBC by intravenous injection of 2×10^6 SRBC. 5 d later, spleen cells from these mice were irradiated (1,200 R) and used as fillers for the limiting dilution analysis of SRBC precursor frequencies of 10-wk-old male and female F₁ spleen cells. Precursor frequencies: (A) Female filler cells, ♀ = 5.0×10^{-5} , ♂ = 5.8×10^{-6} ; (B) Male filler cells, ♀ = 3.8×10^{-5} , ♂ = 3.2×10^{-6} .

B-cell precursors reactive to SRBC. Female mice were chosen as a source of fillers because their spleens contain nearly twice the number of nucleated cells as male F₁ mice. To determine whether the higher precursor frequencies to SRBC for the female responding spleen cells reflected a preferential cooperation of irradiated female filler cells with female B cells, spleen cells from male and female F₁ mice were cultured a limiting dilution with a constant number (3×10^4 cells per culture) of either male or female filler cells. The results (Fig. 5) show that the differences in the male and female frequencies of precursors reactive to SRBC are the same using either male or female irradiated spleen cells as fillers. This is consistent with earlier reports which show all T-cell and macrophage functions in the CBA/N mouse to be normal (2).

Comparison of LPS and SRBC Responses and Surface Ig Expression. In mice expressing the X-linked mutation, there is both a slower acquisition of LPS responsiveness, which requires 4–6 mo to near the level found in normal mice (Figs. 1 and 2), and a lower frequency of precursors reactive to SRBC antigens in vitro (Figs. 3 and 4). The question arises as to how these B-cell responses relate to one another and to the expression of surface Ig in the spleen cell population. To compare B-cell responsiveness to sIg expression, spleen cells from 5-, 10-, and 60-wk-old F₁ mice were tested simultaneously for LPS responsiveness, SRBC-reactive B-cell frequencies, and the percentage of sIg-bearing cells.

The data in Figs. 6 and 7 show the LPS mitogenic and polyclonal responses of

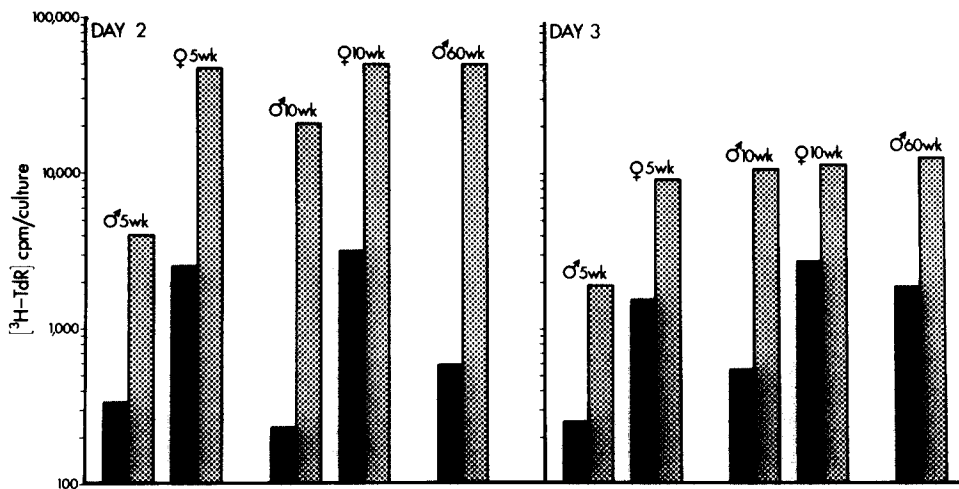


FIG. 6. Effect of age on the LPS mitogenic responses of (CBA/N \times DBA/2) F_1 male and female mice. Spleen cells from 5-, 10-, and 60-wk-old F_1 male and female mice were cultured at 5×10^5 cells per culture in the absence (solid bar) or presence (dotted bar) of $1 \mu\text{g/ml}$ LPS. Cultures were assayed on day 2 or day 3 for incorporation of radioactive thymidine. Data presented are an average of quadruplicate cultures. ■, control; ▨, LPS.

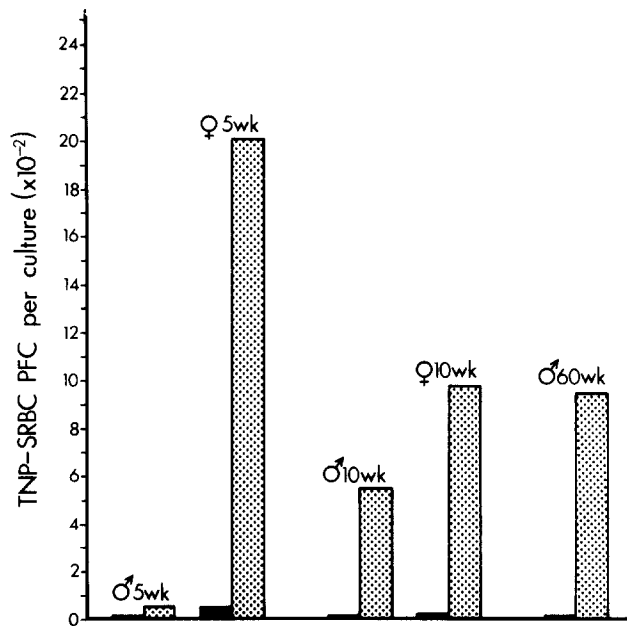


FIG. 7. Effect of age on the LPS polyclonal response of (CBA/N \times DBA/2) F_1 male and female mice. Spleen cells from 5-, 10-, and 60-wk-old male and female mice were cultured at 5×10^5 cells per culture in the absence (solid bar) or presence (dotted bar) of $1 \mu\text{g/ml}$ LPS. Cultures were assayed on day 3 for the number of TNP-SRBC antibody-forming cells per culture. Data presented are an average of quadruplicate cultures. ■, control; ▨, LPS.

spleen cells from these various ages of F_1 mice. Spleen cells from the male F_1 at 5 wk of age gave small responses to LPS which were 12-fold below those of the female F_1 littermate. At 10 wk, spleen cells from the female F_1 showed no increase in LPS responsiveness over that at 5 wk, whereas the male F_1 responses had increased fivefold.

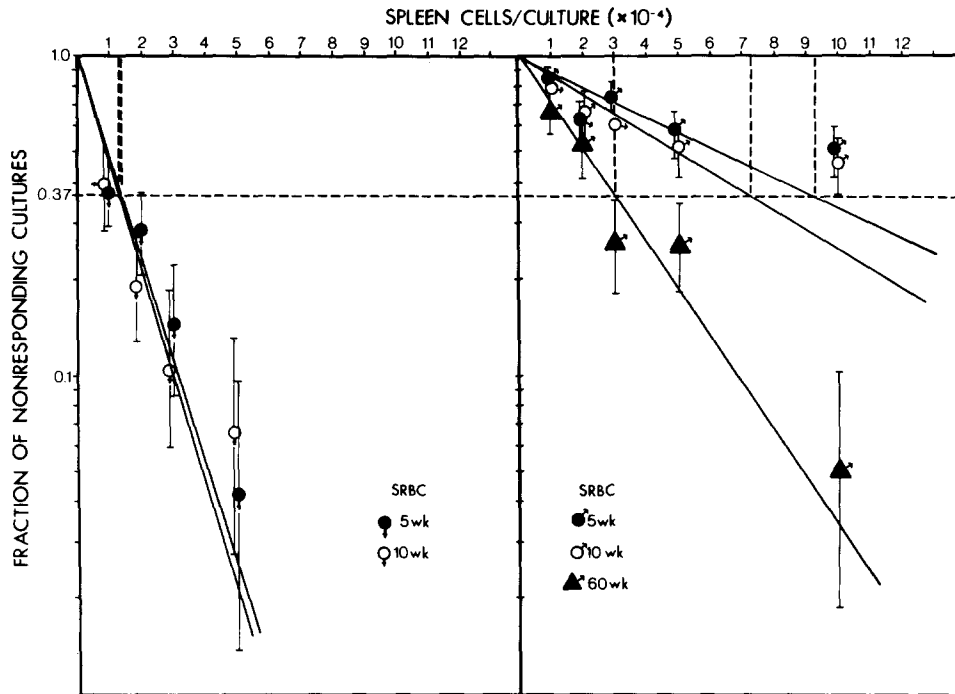


FIG. 8. Effect of age on SRBC-reactive precursor frequencies of (CBA/N \times DBA/2) F_1 male and female mice. Spleen cells from 5-, 10-, and 60-wk-old F_1 male and female mice were cultured at limiting dilution with a constant number of irradiated, SRBC-primed F_1 female spleen cells as fillers. Culture supernates were assayed on day 6 for antibody to SRBC. Precursor frequencies: ♀ 5-wk female, 8.0×10^{-5} ; ♀ 10-wk female, 8.3×10^{-5} ; ♂ 5-wk male, 1.1×10^{-5} ; ♂ 10-wk male, 1.4×10^{-5} ; ♂ 60-wk male, 3.3×10^{-5} .

By 60 wk of age, the spleen cells from the male F_1 showed mitogenic and polyclonal responses equivalent to those of the 10-wk-old F_1 female mouse.

The frequencies of B-cell precursors reactive to SRBC in these mice are shown in Fig. 8. Spleen cells from 5- and 10-wk-old female F_1 mice contained equivalent frequencies of SRBC-specific B cells of 8×10^{-5} and 8.3×10^{-5} , respectively. Spleen cells from male F_1 mice show increasing precursor frequencies of 1.1×10^{-5} , 1.4×10^{-5} , and 3.3×10^{-5} at 5, 10, and 60 wk. The frequency of SRBC-reactive B cells the male mouse at 60 wk was threefold below that of the 10-wk-old female (Fig. 8a). This is in distinct contrast to the LPS responsiveness of these same spleen cell populations, which were equivalent in both the mitogenic and polyclonal assays (Figs. 6 and 7). Mice expressing the X-linked defect, therefore, approach normal levels of LPS responsiveness at between 6 mo and 1 yr of age, but the number of B-cell precursors capable of responding to SRBC remains significantly below normal during the same time period.

One possible explanation for the lower B-cell responses to LPS and SRBC is that the F_1 male spleen cells have many fewer B cells than age-matched females. Previous reports have shown a decreased percentage of B cells in mice expressing the X-linked defect (2, 3, and 27). Surface Ig^+ cells were enumerated in the spleen cell populations of the 5-, 10-, and 60-wk-old mice used in the above experiment. The results in Table I show the young F_1 male mouse to have a substantially lower percentage of sIg^+

TABLE I
Age-related Changes in sIg Expression

Age	sIg ⁺ spleen cells*	
	F ₁ Male	F ₁ Female
<i>wk</i>		%
5	26	48
10	37	45
60	44	ND‡

* Percentage of cells staining with rabbit antisera to total mouse Ig.

‡ Not done.

spleen cells than the age-matched female. The expression of sIg⁺ cells of 5-, 10-, and 60-wk-old males were 26, 37, and 44%, respectively. In contrast, spleens of female mice at 5 and 10 wk contained nearly equivalent numbers of sIg⁺ cells with percentages of 48 and 45, respectively. The appearance of sIg⁺ cells seems to occur in the same time range as acquisition of responsiveness to LPS and SRBC. The less than twofold difference in the percentage of sIg⁺ cells at 5 wk, however, is not enough to account for the >10-fold differences in the responses of these mice to LPS and SRBC. Therefore, the maturation of responsiveness to antigens and mitogens in the CBA/N mouse does not directly correlate with the increase in the gross numbers of sIg⁺ B cells.

Discussion

Since the discovery of the CBA/N defect in 1972, attempts have been made to clearly define the wide variety of changes in expression of B-cell functions and surface markers with only partial success. Certain functions, such as responses to the TI-2 antigen, TNP-Ficoll, have consistently been found to be absent (9, 12, and 14). Responses to LPS and SRBC, however, have varied widely in earlier published reports (1, 2, 7, 10, 14, 16).

The studies in this paper examine closely the effect of age on the expression of various B-cell functions by spleen cells from CBA/N and (CBA/N × DBA/2)F₁ mice. In both strains expressing the X-linked defect (CBA/N and F₁ males), high levels of responsiveness to LPS and SRBC were acquired at a much slower rate than in the phenotypically normal F₁ female. LPS mitogenic responses increase steadily during the first weeks of life to reach normal levels only after 17–52 wk (Figs. 2, 6, and 7). The frequency of B-cell precursors reactive to SRBC in the F₁ male spleens increased during the same time period; however, at 60 wk of age when LPS responses were normal (Figs. 5 and 6), SRBC precursor frequencies were found to still be significantly lower than those of the female F₁ (Fig. 8). The percentage of sIg-bearing spleen cells was also examined and found to increase with the age of the defective mice to normal levels by 60 wk (Table I). Although both sIg expression and responsiveness to LPS and SRBC are lower in defective mice when young, no direct correlation was found between the levels of responsiveness and the number of sIg⁺ cells present in the spleen cell populations.

Spleen cells from defective mice in the age range of 4–6 wk gave LPS mitogenic responses and SRBC precursor frequencies which were 8- to 12-fold below those of the age-matched F₁ females. This is in contrast to the response of these mice to the

thymus-independent antigens TNP-LPS and TNP-BA as reported by Mosier et al. (12). In this report, the responses of spleen cells to TNP-BA and TNP-LPS (TI-1 antigens) appear later in the male F_1 as compared to the female, but at 4–5 wk of age, the male responses were within threefold of the female responses. This apparent difference in the rate of maturation may be a reflection of the different subpopulations of B cells that are responsive to LPS, SRBC, and TI-1 antigens, or may suggest that the external environment of the mouse can affect the rate at which responsive B cells mature in the spleen.

The time range (4–26 wk) during which these defective mice acquire various B-cell functions is a critical one. A general practice in many laboratories is to use mice that are 8- to 12-wk-old for B-cell response assays. Responses of F_1 male and CBA/N mice to LPS and SRBC in this time range have not reached maximal levels and are changing very rapidly. Results from assays using mice in this age range, therefore, can vary greatly from experiment to experiment and from mouse to mouse. As a result, age is an important consideration in the designing of experiments with this strain and in the interpretation of results. Before a function can be definitely classified as defective or absent in the CBA/N strain, the effect of age on the function must be carefully examined.

In view of these data, two categories of B-cell functions in the CBA/N mouse appear to occur: those which never appear and those which gradually accumulate with age. An understanding of the site and action of the defect may come from a close examination of the types of functions which fall into these two categories. Little work has been done with older animals; therefore, few defective responses can be definitely placed in the first category. Smaller spleen size (2), lower background incorporations of radioactive thymidine in spleen cell cultures (Fig. 2), the absence of B-cell colony formation (16), and the lack of response to TNP-Ficoll (12, 14) are characteristics of CBA/N spleen cells that do appear to be absolute. Expression of the CBA/N defect is assumed to be limited to the B-cell compartment of the immune system because T cells, macrophages, and the environment in which the B cells mature all appear to be normal (2, 14). If this assumption is correct, then the above characteristics would be a result of a block in one or more B-cell functions. The smaller spleen size, lower background division in culture, and lack of B-cell colony formation *in vitro* suggest that this block may be in a naturally occurring amplification step somewhere in the latter stages of B-cell ontogeny. An alternate pathway for accomplishing this step could provide a slow accumulation of the functions in the second category without significantly affecting the blocked characteristics listed above.

A much larger number of B-cell defects can be placed in the second category. These include responses to polyclonal mitogens, thymus-dependent antigens and TI-1 antigens (12), and the expression of surface markers such as sIgM/sIgD and Ia (2, 27–29). Of interest is that within this category, there appear to be different levels of defectiveness. Spleen cells from older F_1 male mice, which give equivalent mitogenic responses to LPS as compared to young adult F_1 females, show a significantly lower frequency of SRBC-specific precursor B cells. There are a number of reasons why this may occur. Acquisition of responsiveness to SRBC may be accomplished via a different pathway during ontogeny or may require more steps than does the acquisition of responsiveness to LPS. If this is the case, then LPS responses could reach normal levels at an earlier time than SRBC responses. A second possibility is that the

X-linked defect has varying degrees of effects on mitogen and antigen responses as a result of the different mechanisms by which they signal activation of B cells to form plasma cells. To clarify this point, three examples can be given: (a) spleen cells in the CBA/N mouse have been reported to express a higher density of sIgM (28, 29). The binding of SRBC to a higher number of sIgM molecules on CBA/N B cells may lead to the overproduction of signals which may compete with the signals delivered by the cooperating system (30). This competition could result in a lower number of precursors achieving activation with SRBC, but would have little effect on responses to the polyclonal mitogen, LPS. (b) the CBA/N defect may be in the expression of receptors for helper signals (4), either directly as a result of a mutation in the gene for the receptor, or indirectly as a result of the cells being in a maturation state before receptor expression. In either case, the cells would be less likely to be triggered. (The eventual acquisition of high levels of responsiveness to SRBC argues strongly against the X-linked gene product being the receptor for helper signal.) (c) the defect may lie solely in the driving mechanism for normal B-cell maturation during ontogeny, as discussed earlier. B cells have been shown to mature *in vitro* (31, 32), and SRBC responses of normal spleen cells may involve both the triggering of mature cells and the maturation of less mature cells to a state where they can also be triggered. The absence of this latter situation would result in a lower number of precursors activated in the limiting dilution assays, because only mature cells will respond in the cultures of the defective mice.

The site and action of the CBA/N mutation are not known, but one suggestion has been that the defect is a block in a step or branch of the ontogenic pathway resulting in the deletion of the subpopulation of B cells that are responsive to TI-2 antigens (1, 11). The data presented here, together with the study by Mosier et al. on the ontogeny of TI-1 responses (12), show that the defect also appears to affect the maturation of subpopulations of B cells that are responsive to mitogens, thymus-dependent antigens, and TI-1 antigens. Thus, a simple block in one step of the ontogenic pathway becomes unlikely.

Subpopulation deletion theories have also been used to explain the lowered responsiveness of 6- to 8-wk-old F₁ male mice to LPS (7). In this case, two subpopulations of B cells that are responsive to LPS are hypothesized to exist, one of which is deleted in CBA/N mice. In this paper, we have shown that LPS responses continue to increase past 8 wk of age in the male F₁ mice and will eventually reach the level shown by adult F₁ females (Figs. 6 and 7). In addition, when LPS mitogenic responses of spleen cells from F₁ male and female mice are compared at various culture densities with a wide range of LPS concentrations, the dose-response curves of the cells which do respond in the two spleen cell populations are very similar (Fig. 3). This suggests that the responding B cells in male and female spleens are the same and that the lower response of the F₁ male is a result of a lower frequency of precursors reactive to LPS. We have also shown that 6-wk-old mice are deficient in other B-cell functions such as the frequency of spleen cells reactive to SRBC and the expression of sIg (Fig. 8, Table I). From these findings we conclude that low or variable B-cell responses in young CBA/N or F₁ male mice can be most simply explained by a slower maturation of the B-cell population as a whole and not by a deletion of one or more B-cell subpopulations.

The X-linked mutation of the CBA/N mouse affects a large number of B-cell

functions. The wide variety of effects appear to result either directly or indirectly from X-linked gene(s) controlling the expression of gene products involved in the maturation of B cells. The evidence accumulated so far, however, does not eliminate the possibility that the mutation affects the production of only one gene product which is multifunctional. An understanding of the processes involved in driving normal B-cell differentiation will, hopefully, come from the discovery of the site and action of the CBA/N defect.

Summary

The effect of age on the mitogenic and antigenic responsiveness of B cells is examined in spleen cell cultures of CBA/N and (CBA/N × DBA/2) F₁ mice. Spleen cells from young male F₁ mice (4- to 6-wk old) show lower mitogenic responses to lipopolysaccharide, a lower frequency of sheep erythrocytes (SRBC)-reactive B-cell precursors, and a lower percentage of Ig-bearing cells than age-matched female F₁ mice. The expression of all three functions were found to increase with the age of the F₁ male mice. Whereas male F₁ mice at 60 wk of age showed an equivalent percentage of Ig-bearing spleen cells and a similar mitogenic responsiveness to LPS when compared to adult female F₁ mice, the frequency of SRBC-reactive B-cell precursors remained threefold lower.

These findings reveal that there is a slower maturation of B cells in mice expressing the X-linked defect and suggests that the defect has differential effects on the mechanisms of antigen and mitogen activation of B cells.

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