ROLE OF THE THYMUS IN DIRECTING THE DEVELOPMENT
OF A SUBSET OF B LYMPHOCYTES*

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The study of B lymphocyte heterogeneity, development, and function has been
markedly aided by the availability of mice with the xid-determined immunologic
defect (1). These mice lack a population of late-developing B lymphocytes, which in
normal mice is characterized by the presence of the membrane antigens Lyb-3, 5, and
7. For convenience, we designate such cells as Lyb-5 cells (2–4).

Mice that have the xid-determined defect are unresponsive to a class of antigens
represented by the pneumococcal polysaccharides and hapten derivatives of Ficoll,
dextran, and levan (5–7). These antigens are referred to as type 2 thymus-independent
(TI) antigens. Although capable of stimulating responses in nu/nu mice (8), recent
work indicates that these antigens do indeed require some T cell influences in order
to stimulate in vitro responses (9; J. R. Ketterman and K. Dodd, personal commu-
nication). We will therefore refer to them as type 2 antigens, rather than as type 2 TI
antigens, the designation used previously (10, 11). Mice that express the xid-deter-
mined defect do respond relatively normally to type 1 TI antigens, antigens which
have the property of being polyclonal B lymphocyte activators. Included in this group
are the hapten derivatives of lipopolysaccharide (LPS), Brucella abortus, and Nocardia
water-soluble mitogen (11, 12). Recent studies indicate that these responses may also
be T cell regulated (J. J. Mond, M. Howard, and W. E. Paul, unpublished observa-
tions). Thus, we will designate these antigens simply as type 1 antigens.

The immunologic functions defective in mice with the xid-determined defect have
generally been attributed to Lyb-5 B cells, whereas responses obtained in these mice
are a function of Lyb-5- B cells. These conclusions have been supported in part by
the study of functional properties of Lyb-5- cells from normal mice (13–15).

To examine the possible influence that the thymus and/or T lymphocytes might
have in the development of the Lyb-5- set of B cells, we examined the immunologic
reactivity of nu/nu CBA/N mice, a strain that both expresses the xid-determined

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† Abbreviations used in this paper: BA, Brucella abortus; BSA, bovine serum albumin; FCS, fetal calf serum;
LPS, lipopolysaccharide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sIg,
surface immunoglobulin; TI, thymus independent; TNP, trinitrophenyl.
defect is congenitally athymic, and compared their reactivity with that of CBA/N-+/nu and CBA/Ca-+/nu mice. The latter, although genetically similar to CBA/N has a normal (+) allele at the xid locus. This communication describes the finding that young CBA/N-nu/nu mice (<8 wk of age) show profound defects in B lymphocyte function not found in control mice that express only the xid- or nu/nu-determined genetic defects. A preliminary report of the properties of these mice has already appeared (16).

Materials and Methods

Mice. CBA/N-+/nu and nu/nu and CBA/Ca-+/nu and nu/nu mice were supplied by the Small Animal Section of the Division of Research Services of the National Institutes of Health. The CBA/N-nu/nu congenic strain, which carries both the xid and the nu genes, was prepared by a breeding strategy identical to that described for N:NIH(S)II-nu/nu mice (17).

Antigens. Trinitrophenylated B. abortus (TNP-BA) was prepared as previously described and used at a 10⁻³ dilution of the stock antigen (11). LPS of Escherichia coli 0111:B4 (Difeo Laboratories, Detroit, MI) was used at a concentration of 50 μg/ml.

Cell Culture. A microadaptation (11) of the Mishell-Dutton technique (18) was used to evaluate in vitro antibody production to TNP-BA. Spleen cells (10⁶) were cultured in 0.2 ml of Mishell-Dutton medium containing 10% fetal calf serum (FCS) medium, in flat-bottomed microtiter wells (Costar, Data Packaging, Cambridge, MA) and maintained in a humidified environment of 5% CO₂ and 95% air. Cells were harvested from individual microwells after 4 d of culture, and the number of direct plaque-forming cells (PFC) was assayed by a slide modification of the Jerne hemolytic plaque assay. Target erythrocytes were TNP coupled by the method of Rittenberg and Pratt (19). For evaluation of the proliferative response to LPS, 5 × 10⁵ cells/well were cultured under similar conditions, and [³H]thymidine (6 Ci/mM, 1 μCi/microwell) was added at 48 h of culture. Cells were harvested at 64 h with a MASH II harvester (Microbiological Associates, Walkersville, MD), and incorporation of [³H]thymidine was measured with a liquid scintillation spectrometer (Searle Radiographics, Des Plaines, IL).

Enumeration of Surface Immunoglobulin (sIg) + Cells. Spleen cells were washed and resuspended in complete media containing 0.2% azide and stained with a fluorescein conjugate of affinity-purified rabbit anti-mouse IgM, or with a fluorescein conjugate of 10-4.22, a hybridoma anti-IgD (20), and analyzed on a fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) as previously described (21, 22). The median fluorescence intensity is that channel above and below which lie 50% of the positively stained cells.

Inhibition Radioimmunoassay to Quantitate Serum Ig Concentration. Flexible, round-bottomed polystyrene microtiter plates (Dynatech Laboratories Inc., Dynatech Corp., Alexandria, VA) were coated with purified myeloma proteins from the tumors FLOPC-21 (IgG3,k) or Y5606 (IgG3,k), TEPC-15 (IgA,k), MOPC-21 (IgG1,k), UPC-10 (IgG2a,k), MOPC-195S (IgG2b,k), or TEPC-183 (IgM,k) at a concentration of 10 μg/ml in phosphate-buffered saline (PBS), pH 7.4 for 1–2 h (5.0 μl/well). Purified FLOPC21, Y5606, TEPC15, and TEPC 183 myeloma proteins were purchased from Litton Bionetics, Kensington, MD, and MOPC-21, UPC-10, and MOPC-195S myeloma proteins were purified by protein A-Sepharose affinity chromatography as previously described (23). Following the initial incubation, the wells were washed, and 100 μl of PBS plus 1% bovine serum albumin (BSA) was added to each well for 1 h to reduce subsequent nonspecific protein binding.

The inhibition assay for quantitation of various Ig classes in serum was performed by incubating an aliquot of various dilutions of serum ranging from 1:40 to 1:2,400, or an aliquot of various concentrations of purified myeloma protein ranging from 0.008 μg/ml to 10 μg/ml with an equal aliquot of [³H]goat anti-IgA, [³H]goat anti-IgG1, [³H]goat anti-IgG2a, [³H]goat anti-IgG2b, [³H]goat anti-IgM, or [³H]rabbit anti-IgG3. The diluent used was PBS + 1% BSA + 0.1% sodium azide + 0.005 M disodium ethylene diamine tetraacetate. The labeled antiserum reagent was incubated with the serum dilution or purified Ig for 30 min before transfer to the myeloma protein-coated wells. After a 2–3 h incubation in the wells, the solution containing the labeled antibody was removed, the plates washed 13 times with tap water, and
the amount of $[^{3}H]$labeled anti-isotype reagent bound to each Ig-coated well assayed with the use of a liquid scintillation spectrometer. Standard curves of the inhibition of $[^{3}H]$anti-isotype binding by various concentrations of purified myeloma proteins were constructed through a log/log plot. The standard curves were used to determine the concentration of various isotypes in the various serum samples tested. Six individual serum samples within each group were tested in duplicate to quantitate the amount of Ig in serum from 6-wk-old CBA/Ca nu/nu, CBA/Ca nu/+ , CBA/N nu/nu, and CBA/N nu/+ mice.

**Cell sIg Radiolabeling, Immunoprecipitation, and Analysis.** Methods for cell membrane radioiodination and immunoprecipitation of sIg have been described in detail elsewhere (24). Briefly, $5 \times 10^5$ to $10 \times 10^6$ spleen cells, depleted of erythrocytes, were radioiodinated by lactoperoxidase-catalysis and lysed in Nonidet P-40 detergent. After centrifugation, the clarified lysates were incubated for 1-2 h with a mixture of affinity-purified rabbit anti-$\alpha$, $\mu$, and $\delta$ chain-specific antibodies, followed by precipitation of the immune complexes with 100-150 $\mu$g of 10% fixed protein $A$-bearing *Staphylococcus aureus* (Staph A) bacteria. The precipitates were washed and solubilized with sodium dodecyl sulfate (SDS) buffer.

Samples were reduced in 5% $\beta$-mercaptoethanol and analyzed on vertical polyacrylamide gel slabs in a discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) system (25). The gel slabs were processed for fluorography using the diphenyloxazole/dimethylsulfoxide system (26) and Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY) was exposed to the gel under linear conditions in the presence of DuPont lightning plus intensifying screens (DuPont Instruments, Wilmington, DE), for 12-24 hr. Peak integrations to obtain $\mu\delta$ ratios were performed on the fluorograms using a Zeineh soft-laser scanning densitometer.

**Histological preparation.** Tissue samples of CBA/Ca-+/nu and nu/nu and of CBA/N-+/nu and nu/nu mice were fixed in B-5 and processed by paraffin sectioning for hematoxylin and eosin staining. Sections were made of inguinal, axillary, and mesenteric lymph nodes, and of spleen.

**Results**

**The Failure of TNP-BA and of LPS to Induce Responses by B Cells from CBA/N-nu/nu Mice.** Spleen cells from mice with the nu/nu- or xid-determined defect respond to TNP-BA in vitro with the production of direct PFC of a magnitude comparable to that obtained with cells from their respective normal litter mates. Quite unexpectedly, however, the in vitro anti-TNP-BA response of cells obtained from 6-8 wk-old CBA/N-nu/nu mice was markedly reduced. In the majority of cultures established in microwells, the anti-TNP response to TNP-BA was undetectable (Table I). Even when $10^7$ cells were cultured in larger 35-mm wells, the responses of CBA/N-nu/nu spleen cells was <10% that of CBA/N-+/nu cells, whereas the responses of CBA/Ca nu/nu spleen cells were no different than that of the CBA/Ca-+/nu spleen cells. Anti-TNP-BA responses were evaluated using different amounts of antigen and assayed for anti-TNP-responses on days 3-4 of culture, with the same results (data not shown).

The in vitro proliferative response of such cells to LPS was also severely impaired (Table II). Marked diminution of the proliferative response was seen using all doses of LPS tested (Table III). In most experiments the proliferative responses of CBA/N-nu/nu spleen cells was <10% that of CBA/N-+/nu cells, whereas the responses of CBA/Ca nu/nu spleen cells were no different than that of the CBA/Ca-+/nu spleen cells.

**In Vivo Responses of CBA/N and CBA/Ca (nu/nu) Mice to Intravenous Injection of TNP-BA.** To examine the response of CBA/N-nu/nu mice and -+/nu litter-mate controls to in vivo immunization with a type 1 antigen, TNP-BA was injected intravenously, and anti-TNP-PFC responses were evaluated. Both direct and facilitated anti-TNP-PFC responses of CBA/N-nu/nu mice were significantly diminished as compared with
TABLE I

| Experiment | Cells/well | Anti TNP-PFC/10^6 cultured cells |
|------------|------------|----------------------------------|
|            | CBA/Ca-+/nu | CBA/Ca-nu/nu | CBA/N-+/nu | CBA/N-nu/nu |
| 1          | 10^6        | 326           | 638        | 494        | 0          |
| 2          | 10^6        | 444           | 278        | 147        | 0          |
| 3          | 10^6        | ND†           | 880        | 416        | 0          |
| 4          | 10^6        | ND            | 510        | 405        | 0          |
| 5          | 10^7        | 261           | 300        | 120        | 4          |
| 6          | 10^7        | ND            | ND         | 100        | 18         |

*10^6 spleen cells taken from CBA/N or CBA/Ca-nu/nu and +/nu mice were cultured together with TNP-BA in modified Mishell-Dutton medium. 4 d later cells were harvested and anti-TNP PFC were determined. Results shown represent PFC/10^6 cultured cells after background plaques were subtracted. In experiments 5 and 6, 10^7 spleen cells were cultured in Linbro plates of 35 mm (Linbro Scientific Hamden, CT). In all experiments, pools of spleen cells from two CBA/N-+/nu, CBA/Ca-+/nu, or CBA/Ca-nu/nu mice were used. CBA/N-nu/nu cells from individual animals were cultured separately. The number of CBA/N-nu/nu mice used in experiments 1-6 were 3, 4, 5, 3, 2, and 3, respectively. Results represent geometric mean of triplicate wells using spleen cell pools except for CBA/N-nu/nu mice which were cultured as individual spleens.

† Not done.

TABLE II

In Vitro Proliferative Response of Cells Obtained from CBA/N-nu/nu and CBA/Ca-nu/nu or from Their +/nu Normal Litter Mates to LPS

| Experiment | CBA/Ca-+/nu | CBA/Ca-nu/nu | CBA/N-+/nu | CBA/N-nu/nu |
|------------|-------------|--------------|------------|-------------|
|            | ± SE        | ± SE         | ± SE       | ± SE        |
| 1          | 32,492      | 48,890       | 35,540     | 2,886 ± 1421 |
| 2          | 28,654      | 25,151       | 25,661     | 1,091 ± 326 |
| 3          | ND*         | 37,240       | 36,242     | 656 ± 122   |
| 4          | ND          | ND           | 63,920     | 2,608 ± 509 |

2.5 × 10^5 spleen cells were cultured with 50 μg/ml of E. coli LPS in modified Mishell Dutton medium. At 48 h of culture 1 μCi of [3H]thyminde was added, and 18 h later cells were harvested using a MASH II cell harvester and counted in a liquid scintillation counter. In all experiments pools of spleen cells from two CBA/Ca-+/nu or nu/nu and CBA/N-+/nu were used. CBA/N-nu/nu cells from individual mice were cultured separately. The number of CBA/N-nu/nu mice used in experiments 1-4 were 2, 4, 3, 5, respectively, and results are expressed as the arithmetic mean of the individual responses (±SE).

* Not done.

CBA/N-nu/nu mice (Fig. 1). Whereas the direct anti-TNP-PFC response was reduced by 80%, the indirect PFC response was >90% reduced. In contrast to this, the magnitude of the direct anti-TNP-PFC response of CBA/Ca-+/nu mice was equivalent to that of +/nu mice 4 d after immunization. The facilitated anti-TNP response of CBA/Ca-+/nu mice was comparable to that of +/nu mice early in the response, but did not show the progressive increase in anti-TNP plaques with time after immunization as did the control CBA/Ca-+/nu mice.

Serum Immunoglobulin Levels of CBA/N-nu/nu and CBA/Ca-nu/nu Mice and of Their Normal Litter Mates. Although it appeared that CBA/N-nu/nu mice were profoundly defective in their response to the type 1 antigen TNP-BA and the B cell mitogen LPS, it was conceivable that various other environmental antigens might be more effective in stimulating the immune system in such mice. To evaluate this possibility, serum Ig
TABLE III

In Vitro Proliferative Response of CBA/N-nu/nu and CBA/N-nu/+ Spleen Cells to Various Concentrations of LPS*

| Mitogen added | CBA/N-nu/nu | CBA/N-nu/+ |
|---------------|-------------|------------|
| None          | 776 ± 443   | 2,687 ± 777 |
| LPS 50 μg/ml  | 3,099 ± 317 | 63,812 ± 3,286 |
| 5 μg/ml       | 761 ± 47    | 31,630 ± 3,494 |
| 0.5 μg/ml     | 991 ± 388   | 12,326 ± 2,166 |

* 5 × 10⁵ cells obtained from CBA/N-nu/+ or nu/nu mice were incubated with various concentrations of LPS. 1 μCi of [³H]thymidine was added at 48 h of culture, and 18 h later cells were harvested on the MASH II cell harvester and counted in a liquid scintillation counter.

levels of CBA/N and CBA/Ca-nu/nu and +/nu mice were determined using an inhibition radioimmunoassay (Table IV). As previously described (27, 28), the serum IgM and IgG3 concentrations of euthymic CBA/N mice are significantly reduced as compared with the serum IgM and IgG3 concentrations of CBA/Ca mice, whereas the concentration of the other isotypes were comparable in normal and xid mice. In marked contrast to this, the concentrations of all classes of serum Ig were reduced in
**TABLE IV**

Serum Ig Levels of CBA/N-+/nu and CBA/Ca-+/nu Mice and of Their Normal +/nu Litter Mates

| Serum Immunoglobulin Concentration (µg/ml)* | IgM | IgG | IgG1 | IgG2b | IgG2a | IgA |
|-----------------------------------------------|-----|-----|------|-------|-------|-----|
| CBA/N-+/nu (n = 6)                            | 257 ± 24 | 24 ± 7 | 250 ± 37 | 1,094 ± 110 | 796 ± 76 | 65 ± 10 |
| CBA/N-+/nu (n = 6)                            | 52 ± 15 | 0.01 | 16 ± 9 | 8 ± 4 | 16 ± 7 | 1.3 ± .7 |
| CBA/Ca-+/nu (n = 6)                           | 1,185 ± 41 | 215 ± 34 | 484 ± 95 | 850 ± 185 | 724 ± 45 | 61 ± 7 |
| CBA/Ca-+/nu (n = 6)                           | 1,208 ± 64 | 252 ± 62 | 128 ± 39 | 373 ± 77 | 807 ± 95 | 31 ± 5 |

* Serum Ig levels were quantitated through the use of an inhibition radioimmunoassay. Various dilutions of each serum were tested for their ability to inhibit the binding of [3H]-labeled anti-isotype reagents to myeloma-coated microtiter wells. The amount of inhibitory Ig of each class was quantitated through the use of standard curves established from the appropriate myeloma inhibitors. Results are expressed as mean µg/ml concentration of six individual sera ±SE.

**TABLE V**

FACS Analysis of sIgM* and of sIgD* Spleen Cells of CBA/N-+/nu and CBA/Ca-+/nu Mice and of Their Normal +/nu Litter Mates†‡

| Donor cells‡ | Age | Percent sIgM* cells | Median fluorescence intensity | Percent sIgD* cells | Median fluorescence intensity |
|--------------|-----|---------------------|------------------------------|---------------------|------------------------------|
| CBA/Ca-+/nu  | 4   | 56.3                | 49                           | 58.7                | 46                           |
| nu/+         |     | 48.1                | 39                           | 50.7                | 59                           |
| CBA/N-+/nu   | 4   | 46.0                | 100                          | 31.6                | 21                           |
| nu/nu        |     | 44.5                | 101                          | 27.6                | 20                           |
|              |     | 42.1                | 98                           | 41.6                | 43                           |
| CBA/Ca-+/nu  | 6   | 56.3                | 49                           | 62.8                | 61                           |
| nu/+         |     | 36.8                | 36                           | 33.9                | 67                           |
| CBA/N-+/nu   | 6   | 44.0                | 85                           | 31.7                | 26                           |
| nu/nu        |     | 49.7                | 95                           | 32.2                | 26                           |
|              |     | 35.0                | 76                           | 34.9                | 87                           |

* 10⁶ cells obtained from various donor mice were washed and prepared for analysis on the FACS after staining with FITC-10-4.22 (anti-δ) or FITC anti-µ. The percent of sIgD* and sIgM* cells were enumerated, and the median fluorescence intensity of the positive cells was determined.
† Each group consists of a pool of two mice.

CBA/N-+/nu mice as compared with that seen in CBA/N-+/nu or in CBA/Ca-+/nu mice. Indeed, the concentration of IgM, which at 52 µg/ml was the most abundant isotype in CBA/N-+/nu serum, was only 20% of the CBA/N IgM serum concentration and ~5% of CBA/Ca IgM serum concentration. All other Ig classes were found at concentrations of 16 µg/ml or less in CBA/N-+/nu serum.

**FACS Analysis of sIgM B Lymphocytes in CBA/N-+/nu Mice and SDS-PAGE Quantitation of the Expression of sIgD and sIgM.** To evaluate whether the diminished responses of CBA/N-+/nu mice was a reflection of reduced numbers of B lymphocytes in such mice, spleen cells from CBA/N-+/nu, CBA/Ca-+/nu mice, and of their +/nu litter mates were stained with fluorescein conjugates of anti-µ and anti-δ antibodies and analyzed on the FACS (Table V). At both 4 and 6 wk of age, the fraction of sIgM+...
cells in CBA/N-\textit{nu/nu} mice was \(~45\%\), a value comparable to that of CBA/N mice. As described previously (29), the median fluorescence intensity of the IgM positive CBA/N cells was significantly higher than that of CBA/Ca B cells, reflecting an increased expression of sIgM on the B cells of CBA/N mice. CBA/N-\textit{nu/nu} cells also displayed this increased expression of sIgM on their B cells. Despite the relatively normal fraction of sIgM\textsuperscript+ B lymphocytes in CBA/N-\textit{nu/nu} mice, the absolute number of B lymphocytes in the spleens of CBA/N-\textit{nu/nu} mice was three- to fourfold lower.

### Table VI

| Cells used \* | Age wk | sIgD/sIgM \(\dagger\) |
|--------------|--------|----------------------|
| CBA/N-\textit{nu/nu} | 5 | 0.38 |
| \textit{nu/+} | | 1.14 |
| CBA/Ca-\textit{nu/nu} | 5 | 1.80 |
| \textit{nu/+} | | 1.94 |

\* Spleen cells from two or three mice of the indicated strains were radioiodinated, immunoprecipitated, and subjected to SDS-PAGE analysis as described in Materials and Methods.

\(\dagger\) The area under sIgD and sIgM peaks was determined as described in Materials and Methods, and the ratio between the two calculated.

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**Fig. 2.** Lymph nodes from adult (8-12-wk) mice; initial magnification \(\times40\). A, CBA/Ca\textit{+/-}\textit{nu}; B, CBA/N\textit{+/-}\textit{nu}; C, CBA/Ca\textit{-nu}\textit{/nu}; D, CBA/N\textit{-nu}\textit{/nu}.
than that of CBA/N-+/nu mice, because the total number of spleen cells was reduced by this fraction (data not shown).

There was a significant excess in the percentage of cells that were sIgM* over sIgD* in the spleens of 4- and 6-wk-old CBA/N-+/nu mice, which was not observed in CBA/Ca mice. This was consistent with approximately one third of the cells from CBA/N-+/nu mice of both 4 and 6 wk of age, being sIgM* sIgD-. Furthermore, the median fluorescence intensity of IgD on sIgD* cells of CBA/N-+/nu mice was two-to threefold less than that of cells obtained from CBA/N-+/nu mice, and there was no increase in the intensity of staining with anti IgD between 4 and 6 wk of age of the CBA/N-+/nu mouse despite the observed twofold increase in median fluorescence intensity of sIgD* cells from 6-wk-old +/-nu litter mates.

To further quantitate the expression of sIgD and sIgM on cells from CBA/N-+/nu mice, cells were radiolabeled by lactoperoxidase catalyzed iodination followed by SDS-PAGE (Table VI). It is apparent that the IgD/IgM ratio on the cells obtained from CBA/N-+/nu mice is substantially less than that of CBA/Ca-+/nu or nu/nu cells, as previously reported (30). However, the IgD/IgM ratio from CBA/N-+/nu cells is strikingly lower than that of the +/-nu litter mates.

**Histology of CBA/N-+nu Peripheral Lymph Node.** Examination of sections of peripheral lymph nodes of CBA/N-+nu/nu mouse revealed the virtual absence of lymphocytes; the only cells identifiable in these sections were stromal cells (Fig. 2 D). Sections of lymph nodes from CBA/Ca-+/nu mice showed the presence of lymphocytes only in the far cortex; primary follicles without germinal centers were scattered throughout this area. The paracortex and medulla were devoid of lymphocytes (Fig. 2 C). CBA/N-+/nu lymph nodes (Fig. 2 B) were similar to those of CBA/Ca-+/nu mice in that they exhibited well-developed germinal centers, but appeared to have decreased numbers of small mantle layer lymphocytes surrounding the germinal centers, and a normal distribution of lymphocytes. These unusual histologic findings resemble those previously reported for NIH(s)II-nu/nu mice (17) (which expresses the xid defect), as do the abnormalities observed in the spleens of CBA/N-+nu/nu mice.

**Discussion**

There is little evidence from the murine system that the thymus plays a role in the development of B lymphocytes, because by all measures the function of B lymphocytes from nu/nu mice is normal. However, because many of the functions tested in nu/nu mice are presumably the result of the activation of Lyb-5+ cells, it was of interest to study the functional capacity of B lymphocytes of nu/nu mice that expressed the xid defect, and thus lacked Lyb-5+ cells. If the thymus played no role in B lymphocyte development, one would expect that the functional properties of the B lymphocytes from such mice to be similar to those of CBA/N-+nu B lymphocytes. However, when tested in vitro, the spleen cells of CBA/N-+nu/nu mice showed markedly reduced responsiveness to LPS and to TNP-BA when compared to the spleen cells of CBA/N-+/nu or CBA/Ca-+/nu mice. Similar findings were obtained in in vivo studies of the responsiveness of CBA/N-+nu mice to TNP-BA.

In addition to their low responsiveness to type 1 antigens, CBA/N-+nu/nu mice showed a >80% diminution in the concentration of serum IgM and a >95% diminution in the serum concentrations of each of the IgG classes and IgA when compared with CBA/Ca-+nu/nu mice. Thus, unlike CBA/N-+nu mice who have relatively
normal serum concentrations of IgG₁, IgG₂a, IgG₂b, and IgA and lower levels of IgM and IgG₃, CBA/N-𝑢/𝑢 mice have reduced levels of all classes of serum Ig. These concentrations are significantly lower than those seen in germ-free mice (31), suggesting that CBA/N-𝑢/𝑢 mice fail to respond to environmental antigens. Despite these low serum Ig levels, these mice have a mortality similar to that of CBA/Ca-𝑢/𝑢 mice, under laboratory conditions. The abnormally low responses of CBA/N-𝑢/𝑢 mice to TNP-BA or LPS and their inability to respond to environmental antigens indicates that the B lymphocytes of mice that express the xid defect do not function normally in the absence of a thymic influence.

Because it appears that the thymus plays an important role in influencing the development of a class of B lymphocytes, it was of interest to determine whether this influenced the frequency of slg⁺ cells or their maturational status. The average number of lymphocytes in the spleens of CBA/N-𝑢/𝑢 mice was $3 \times 10^7$ to $4 \times 10^7$, whereas the number of cells from CBA/N-+/𝑢 spleens was $9 \times 10^7$ to $10 \times 10^7$. The maturational status of B lymphocytes from these mice was initially studied using radiodination, immunoprecipitation followed by SDS-PAGE analysis. The ratio of surface p vs. δ on the B lymphocytes of CBA/N-𝑢/𝑢 mice was considerably higher than on B lymphocytes of CBA/N-+/𝑛𝑢 mice at 6 wk of age. The expression of B lymphocyte slgM and slgD was further studied using the FACS. No differences were noted in the frequency of slgM⁺ cells or in their median fluorescence intensity as compared to CBA/N-+/𝑛𝑢 cells. The increase in median fluorescence intensity of slgM on these cells as compared with either normal 𝑢/𝑢 or normal +/𝑛𝑢 mice reflects the immaturity of these cells and is a result of the xid defect (29). However CBA/N-𝑢/𝑢 B lymphocytes did not resemble those of CBA/N-+/𝑛𝑢 mice in that the frequency of slgM⁺ slgD⁻ cells was increased, and represented a significant proportion of B lymphocytes of CBA/N-𝑢/𝑢 at both 4 and 6 wk of age. Furthermore, those CBA/N-𝑢/𝑢 cells that were slgD⁺ expressed significantly less slgD that

![Diagram](image)

**Fig. 3.** Proposed scheme of B lymphocyte development. The population of immature Lyb-5⁻ B cells contains precursors that develop into mature Lyb-5⁺ B cells and others that develop into mature Lyb-5⁻ B cells. The former developmental pathway is independent of T cell influence, whereas the latter is dependent on T cell or thymic regulation. Mature Lyb-5⁺ B cells are found in normal adult mice and in adult 𝑢/𝑢 mice of normal strains. Mature Lyb-5⁻ B cells are found in normal adult mice and in mice with the xid defect (i.e., CBA/N mice). Immature Lyb-5⁻ B cells are found in neonatal normal mice and in congenitally athymic xid mice (i.e., CBA/N-𝑢/𝑢 mice).
CBA/N-+/nu cells as reflected in their low median fluorescence intensity. Thus, the increased ratio of incorporated radioactivity in δ versus μ of CBA/N-μ/μ/nu B lymphocytes reflects both a decreased number of slgD+ cells and a reduced surface expression of slgD on these cells, as well as an increased expression of slgM. This phenotype is characteristic of the B lymphocytes of very immature normal or CBA/N mice (32–34).

In the course of these experiments we found that CBA/N-μ/μ mice >12 wk of age sometimes displayed greater responses to TNP BA and LPS than did mice 6–8 wk of age. Thus the profound B cell defect seen in animals expressing the μ and xid genes may be more of a delay in B cell maturation than a complete block. It is possible that the increase in responsiveness of older CBA/N-μ/μ mice may reflect the presence of increased numbers of T cells in these mice as has been reported in older strains of nude mice (35). Additionally, it has been reported that development of immature thymocytes can proceed in the absence of direct thymus influence (36) and may be accelerated in the presence of T cell derived factors (37) as well as by LPS conditioned media (38). Alternatively, there may be other, less efficient pathways for B cell development that are influenced solely by macrophages or other cellular and humoral factors.

The data presented here suggest that a thymic influence is critical for the normal development of mature Lyb5- B lymphocytes from their precursors and that the development of Lyb5+ B lymphocytes, present in CBA/N-μ/μ mice but not in CBA/N-μ/μ mice, is not dependent (or is less dependent) on a thymic influence. If this were the case, it would imply that mature Lyb-5- and Lyb-5+ B lymphocytes arise along independent paths of cell differentiation (Fig. 3). Another possibility might well be that the Lyb5- B lymphocytes of the CBA/N mouse are not equivalent to Lyb-5- B lymphocytes of normal adult mice, and it is only the former population of cells that require a thymic influence, whereas the latter population develops in a normal fashion in the absence of a thymus.

Summary

In an effort to evaluate the role of the thymus in influencing the development of Lyb-5- B lymphocytes, mice expressing both the xid and μ gene defects were studied. Mice expressing either of these defects respond to both trinitrophenylated Brucella abortus and lipopolysaccharide; whereas mice with the combined defect show markedly suppressed responses. The other abnormalities included: (a) >80 percent diminution of serum Ig levels; (b) significant increase in the number of slgM+ slgD- B lymphocytes; (c) reduced expression of IgD on slgD+ cells; and (d) a strikingly abnormal histology of their lymphoid tissue. Because μ/μ mice that do not express the xid defect appear relatively normal, it would suggest that the development of Lyb-5- B lymphocytes require a thymic influence for normal maturation, whereas, Lyb-5+ B lymphocytes are relatively independent of such influences.

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