ESTABLISHMENT OF AN INBRED LINE OF MICE THAT EXPRESS A SYNERGISTIC IMMUNE DEFECT PRECLUDING IN VITRO RESPONSES TO TYPE 1 AND TYPE 2 ANTIGENS, B CELL MITOGENS, AND A NUMBER OF T CELL-DERIVED HELPER FACTORS*

BY JAMES J. MOND, GERARD NORTON, WILLIAM E. PAUL, IRWIN SCHER, FRED D. FINKELMAN, SHIRLEY HOUSE, MARY SCHAFFER, PATRICIA K. A. MONGINI, CARL HANSEN, AND CONSTANTIN BONA

From the Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; the Department of Microbiology, Mount Sinai School of Medicine, New York 10021; the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases and the Division of Research Services, Veterinary Research Branch, National Institutes of Health, Bethesda, Maryland 20205; the Department of Immunology, Naval Medical Research Institute, Bethesda, Maryland 20814; and the Hospital for Joint Diseases Orthopedic Institute, New York 10003

Strains of animals with genetic defects in their immune system have greatly facilitated our understanding of various pathways in B and T lymphocyte development. Only a limited number of mouse strains exist that express relatively well-defined genetically determined defects in B lymphocyte function. Particularly notable among these are CBA/N mice, which possess a defective X-linked gene, xid, which interferes with the development of cells that have the Lyb5 surface antigen. Such mice are unable to respond to purified polysaccharide antigens (1, 2). We have recently shown that introduction of the nu/nu gene into mice with the xid defect further impairs their ability to respond to B cell stimulants (3). The resultant CBA/N nu/nu mouse shows an immune defect that is greater than would be predicted from the combined defect of the two parental strains. Investigations of immune responses using this strain of mouse suggested that the T cells may play an important role in directing the maturation of Lyb5- B cells. Since it appeared that combinations of genetically defined immune defects can be expressed synergistically rather than additively, we were interested in investigating the immune responses of mice that express the xid-determined defect and possess a defective gene or genes found in the C3H mouse strain.

We have previously reported (4) that when (CBA/N × C3H/HeJ)F1 female mice, which are heterozygous for the xid gene (+/xid), are crossed to male C3H/HeJ mice, B cells of half of the male progeny were unable to make in vitro

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immune responses to type 1 antigens and to B cell mitogens that could effectively stimulate cells of both CBA/N and C3H/HeJ type (4). Since similar results were obtained using C3H/HeN mice as the breeding partners, it appeared that the LPS-determined defect of the C3H/HeJ mice was not required to produce these synergistic defects. We have now established two inbred lines of mice that express this synergistic immunological defect and we report here the immunological features of these mice. These strains possess the xid trait on either the C3H/HeN or C3H/HeJ backgrounds and are designated C3.CBA/N and C3J.xid, respectively. Spleen cells from mice of both strains do not respond in vitro to any B cell mitogen we have examined, including lipopolysaccharide (LPS), Nocardia water-soluble mitogen (NWSM), and antimmunoglobulin (anti-Ig) antibody conjugated to Sepharose beads. Additionally, their in vitro antibody response to the type 1 antigen trinitrophenyl-Brucella abortus (TNP-BA) is either undetectable or 10–100 times lower than that of cells from parental strains. Responsiveness of spleen cells from these defective strains cannot be enhanced by a T cell factor-rich supernatant, whereas responsiveness of cells from both C3H and CBA/N strains can be enhanced. F1 female mice resulting from the mating of C3.CBA/N and C3J.xid exhibit the same profound immunologic defect as their parents, suggesting that the genetic basis for their defects is identical and noncomplementary.

Materials and Methods

Mice. C3J.xid mice were obtained from original crosses of CBA/N (xid/xid) females (obtained from the Division of Research Services, National Institutes of Health) with C3H/HeJ (+/Y) males (The Jackson Laboratory, Bar Harbor, ME). F1 females resulting from this cross, which are heterozygous at the xid locus (+/xid), were crossed to C3H/HeJ males. The male offspring of this cross were immunized with TNP-aminoethylcarbamylmethyl-Ficoll (TNP-Ficoll) (20 μg), and their sera were tested 5 d later for the presence of anti-TNP antibodies by hemagglutination of TNP-sheep erythrocytes (TNP-SE). Mice that failed to produce anti-TNP antibody were judged to be xid/Y and were mated to C3H/HeJ females. Four additional cycles of this type were carried out. xid/Y males from the ninth backcross generation were mated to +/xid females from the eighth backcross generation. Male and female progeny from this cross that were unresponsive to TNP-Ficoll were mated to create an inbred strain. Tail skin from mice of the fifth intercross generation was grafted to C3H/HeJ, BALB/c, and CBA/N mice. The grafts were not rejected by C3H/HeJ mice over a 4-mo period whereas BALB/c mice rejected grafts by day 10 and CBA/N mice rejected the grafts by day 21. C3.CBA/N mice were bred as follows: The xid gene was introduced into the C3H/HeN inbred mouse strain by repeated cycles of backcrossing. The initial cross consisted of mating a CBA/N female, which is homozygous for the xid gene, to a C3H/HeN male. The female progeny from this mating were heterozygous for the xid gene and the males, hemizygous. One of the male offspring was backcrossed to a CBA/N female. The progeny from the backcross mating were either homozygous, in case of the females, or hemizygous, for the males. The second cycle consisted of mating a homozygous female, now designated as C3.CBA/N, to a C3H/HeN male. A male offspring from this mating was backcrossed to his mother to produce a homozygous female that was bred to a C3H/HeN male. This procedure was repeated for

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1 Abbreviations used in this paper: BA, Brucella abortus; CE, chicken erythrocyte; Con A, concanavalin A; FITC, fluorescein isothiocyanate; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NWSM, Nocardia water-soluble mitogen; PFC, plaque-forming cell; PMA, phorbol myristic acetate; POL, polymerized flagellin; SIII, type III pneumococcal polysaccharide; SE, sheep erythrocyte; slg, surface immunoglobulin; TNP, trinitrophenyl.
the subsequent cycles that currently continue. The N equivalents for the mice used in this study vary from 5 to 7.

**Antibody-dependent Cell-mediated Cytotoxicity.** Antiserum to chicken erythrocyte (CE) was prepared by injecting a 10% suspension of CE into New Zealand rabbits. A pool of sera from three rabbits was used that had a hemagglutinin titer of $1.6 \times 10^4$. CE target cells ($10^7$) were incubated for 1 h at 37°C with 150 nCi of sodium-51chromate ($\text{Cr}^{51}$) (sp act 264 mCi/mg) (New England Nuclear Boston, MA) and washed three times before use. Spleen cells from C3J.xid, C3H/HeJ, and CBA/N mice were used as effector cells. 2.5 $\times 10^6$ effector cells in 1 ml RPMI 1640 medium supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, VA) were mixed with $10^5$ labeled CE in 0.1 ml of heat-inactivated rabbit anti-CE antiserum, diluted 1:3,200, and then incubated at 37°C for 18 h. Effector cells cultured with $\text{Cr}^{51}$-labeled CE in the presence of normal rabbit serum at the same dilution with $\text{Cr}^{51}$-labeled CE alone in the absence of serum served as controls. Total radioactivity was taken to be equivalent to the radioactivity released by incubating the target cells with 1% Triton X-100. The tubes were incubated in an upright position at 37°C in an atmosphere of 5% CO$_2$. They were then centrifuged for 10 min at 160 g and the radioactivity of the supernatant was determined in a gamma counter.

Cytotoxicity was expressed as percent specific release of label as follows: Percent cytotoxicity = \[ \frac{(\text{cpm}_\text{Ab} - \text{cpm}_\text{R})/\text{cpm}_\text{T} - \text{cpm}_\text{R})}{\text{cpm}_\text{T}} \times 100 \] where $\text{cpm}_\text{Ab}$ is the counts in supernatant of the tubes with rabbit serum (normal or anti-CE), $\text{cpm}_\text{R}$ is the spontaneous release of counts in the control tubes with nonimmune serum, and $\text{cpm}_\text{T}$ is the total radioactivity released by treatment with Triton X-100.

**Antigens, Mitogens, and T Cell Factors.** TNP-Ficoll and TNP-BA were prepared as previously described (5, 6). LPS from Escherichia coli 0111:B4, prepared according to the Boivin procedure, was purchased from Difco Laboratories, Inc., Detroit, MI. Since this mitogen contains lipoprotein, it is able to stimulate proliferative and plaque-forming cell (PFC) responses by C3H/HeJ spleen cells. NWSM was prepared according to the technique of Clorbovn et al. (7). Concanavalin A (Con A) was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Polymerized flagellin (POL) from Staphylococcus adelaide and type III pneumococcal polysaccharide (SIII) were a gift from Dr. P. Baker, Laboratory of Microbial Immunity, NIH. Supernatant fluid from the EL4 thymoma line, stimulated with phorbol myristic acetate (PMA), was collected as previously described (8). Goat anti-mouse IgM and IgD antibodies were purified and conjugated to Sepharose beads as previously described (9, 10).

**In Vitro and In Vivo Responses to Type 1 and Type 2 Antigens and to B Cell Mitogens.** Spleen cells from control or experimental mice were cultured in flat-bottomed microtiter plates (Costar, Data Packaging, Cambridge, MA) at a density of $10^6$ cells per 0.2 ml of modified Mishell-Dutton medium together with TNP-Ficoll ($5 \times 10^{-4}$ µg/ml) or TNP-BA ($10^{-5}$ dilution of stock) (3). Anti-TNP direct PFC responses were measured after 3 or 4 d of culture by a modified Jerne hemolytic plaque assay with TNP-SE as the target cells. Proliferative responses were measured by culturing $5 \times 10^5$ or $2.5 \times 10^6$ spleen cells in microtiter wells with LPS (50 µg/ml), NWSM (50 µg/ml), POL (100 µg/ml), SIII (100 µg/ml), or Con A (2 µg/ml). 1 µCi of $[^{3}H]$thymidine (sp act 20 Ci/m mole) was added on day 2 of the culture period and uptake of $[^{3}H]$thymidine was determined 18 h later using a liquid scintillation spectrometer.

**Cell Separation Procedures.** Mice received injections of 0.04 ml of rabbit anti-mouse thymocyte antiserum (ATS) (Microbiological Associates, Walkersville, MD). 48 h later, spleen cell suspensions were prepared and treated with monoclonal anti-mouse Thy-1.2 (Becton, Dickinson & Co., Sunnyvale, CA) and guinea pig complement and then resuspended to $10^7$ cells/ml in RPMI supplemented with 10% FCS. 5.0 ml of these cells, at $10^7$/ml, were incubated for an additional 1 h at room temperature on petri dishes coated with goat anti-mouse Ig antibody; adherent cells were recovered by detachment with a rubber policeman. Nonadherent cells (null cells) were found to contain $<2\%$ surface Ig (sIg)$^+$ or Thy-1.2$^+$ cells, as evaluated by analysis of cells stained with fluorescein isothiocyanate (FITC)-labeled monoclonal anti-IgM antibody or FITC-labeled anti-Thy-1.2 antibody, using fluorescence microscopy.
For in vivo immunization, 0.1 ml of a 1:10 dilution of TNP-BA was injected intravenously and anti-TNP PFC responses measured 5 and 7 d later. Sera was assayed for anti-TNP antibody using a radioimmunoassay as previously described (11).

Flow Microfluorometric Analysis of slg+ Cells. FITC conjugates of F(ab')2 fragments of rabbit anti-mouse IgM, goat anti-mouse IgD, and goat anti-keyhole limpet hemocyanin (KLH) were prepared as previously described (10). FITC-conjugated anti-Thy-1.2 was prepared using a monoclonal anti-Thy-1.2 (Becton, Dickinson & Co.). Preparation of cells for staining and the method for their analysis on the FACS II (B-D FACS Systems, Becton, Dickinson & Co.) has been previously described (10).

Results

In Vitro Antibody Responses of C3J.xid and C3.CBA/N to TNP-BA and TNP-Ficoll. We have recently proposed that antigens be classified on the basis of their ability to stimulate antibody responses in nu/nu and xid mice (12, 13). Type 1 antigens, which include TNP conjugates of B. abortus, LPS, and NWSM, have

| Experiment No. | Mouse strain (number of mice) | Anti-TNP PFC/culture |
|----------------|--------------------------------|----------------------|
| 1              | CBA/N (3)                      | 164 (1.14)           |
|                | C3H/HeN (3)                    | 298 (1.21)           |
|                | C3.CBA/N (3)                   | 0                    |
| 2              | CBA/N (1)                      | 251 (1.02)           |
|                | C3H/HeN (1)                    | 471 (1.28)           |
|                | C3.CBA/N                       | 20 (1.05)            |
|                | No. 1                          | 40 (1.11)            |
|                | No. 2                          | 0                    |
|                | No. 3                          | 34 (1.41)            |
|                | No. 5                          | 20 (2.43)            |
|                | No. 6                          | 10 (1.10)            |
| 3              | CBA/N (2)                      | 166 (1.20)           |
|                | C3.CBA/N (5)                   | 6 (1.52)             |
| 4              | CBA/N (2)                      | 100 (1.24)           |
|                | C3H/Hej (2)                    | 398 (1.31)           |
|                | C3J.xid                        | 0                    |
|                | No. 1                          | 0                    |
|                | No. 2                          | 0                    |
|                | No. 3                          | 0                    |
|                | No. 4                          | 0                    |

10⁶ spleen cells from normal or immune-defective mice were cultured with TNP-BA, and anti-TNP PFC were assayed on day 3 or 4 of culture. Results represent geometric mean of triplicate wells (x/- mean SE). In experiments 1 and 3, cells were pooled from groups of mice (number indicated in parenthesis) and pooled cells were cultured. In experiment 2, cells from individual mice were cultured. In experiment 4, pools of CBA/N and C3H cells were cultured; cells from individual C3J.xid mice were cultured.
**TABLE II**

Addition of C3J.xid Spleen Cells to C3H Spleen Cells Does Not Suppress the In Vitro Response to TNP-BA

| Cells in culture | Anti-TNP PFC/culture |
|------------------|-----------------------|
|                  | Exp. 1 | Exp. 2 | Exp. 3 |
| C3H              | 506 (1.25) | 74 (1.34) | 56 (1.11) |
| C3J.xid          | 9     | 0     | 0     |
| C3H + C3J.xid    | 798 (1.23) | 210 (1.27) | 94 (1.14) |

C3H cells were cultured at $10^6$, $5 \times 10^5$, or $4 \times 10^5$ cells/well in experiments 1–3, respectively, together with $5 \times 10^5$ C3J.xid cells and TNP-BA. 3 d later anti-TNP PFC were determined. Results represent the geometric mean ($\times \pm$ mean SE) of triplicate cultures.

been shown to stimulate in vitro and in vivo responses by $nu/nu$ mice and by CBA/N xid mice. Type 2 antigens, represented by soluble polysaccharides and their hapten conjugates, stimulate responses in $nu/nu$ mice but not in CBA/N mice. While spleen cells from C3H/HeN, C3H/HeJ, and CBA/N mice respond to the type 1 antigen TNP-BA, splenic B cells from the two inbred lines described here, C3.CBA/N and C3J.xid, were either poorly responsive or unresponsive to both antigens (Table I). This low level of responsiveness was seen at all cell densities tested varying from $2.5 \times 10^5$ to $10^6$ per well (data not shown).

The impaired responsiveness of cells from the C3J.xid mice does not appear to be a consequence of the presence of naturally occurring suppressor cells, since equal mixtures of these nonresponder spleen cells with C3H spleen cells did not suppress the antibody response to TNP-BA mounted by the normal spleen cell population (Table II). In other experiments, removal of T cells from the C3J.xid spleen cell population did not lead to increased responsiveness of their B cells to TNP-BA, further suggesting that a T cell suppressor population was not influencing their lack of responsiveness to TNP-BA. Addition of T cells from C3H/HeN mice to cultures of spleen cells from C3.CBA/N also did not influence their responsiveness to TNP-BA (data not shown).

**Mitogen Responsiveness of Cells from C3.CBA/N and C3J.xid Mice.** In vitro proliferative responses to the B cell mitogens LPS and NWSM, which previously have been shown to stimulate responses by B cells of CBA/N, C3H/HeJ, and C3H/HeN mice, were evaluated in spleen cell cultures of C3.CBA/N and C3J.xid mice. In the experiments reported here these mitogens stimulated substantial proliferation by spleen cells from control C3H/HeN, C3H/HeJ, and CBA/N mice as evaluated by incorporation of $[^3H]$thymidine. LPS and NWSM had either minimal or no stimulatory effect on spleen cells from C3.CBA/N and C3J.xid mice (Table III). To evaluate whether the occasional meager responses stimulated by NWSM reflected stimulation to the Ig$^+$ cell population of C3J.xid mice, the responses of unseparated spleen cell populations were compared with those of purified sIg$^+$ cells or of sIg$^-$ Thy-1.2$^+$ cells at various cell concentrations. The modest response of C3J.xid spleen cells to NWSM appeared to be almost completely accounted for by the proliferative responses mounted by the null cell population. The purified B cell population of these mice was essentially unresponsive to this mitogen (Fig. 1). Previous studies using rabbit lymphocytes also
Table III

| Exp | Strain (number of mice) | Medium | LPS (Boivin) | NWSM | Con A |
|-----|-------------------------|--------|--------------|------|-------|
| 1   | C3H/HeN (3)             | 5,293  | 29,332       | 16,606| 78,242 |
|     | CBA/N (3)               | 5,401  | 44,482       | 29,880| 60,135 |
|     | C3.CBA/N (3)            | 1,652  | 3,849        | 4,896 | 90,438 |
| 2   | C3H/HeN (1)             | 8,337  | 24,910       |       | 98,217 |
|     | CBA/N (1)               | 422    | 41,364       |       | 180,295|
|     | C3.CBA/N               |        |              |      |       |
|     | No. 1                   | 2,299  | 3,965        | 152,295|
|     | No. 2                   | 2,130  | 4,125        | 155,428|
|     | No. 3                   | 1,581  | 5,716        | 65,581 |
|     | No. 4                   | 2,799  | 4,319        | 137,478|
|     | No. 5                   | 1,636  | 5,432        | 141,295|
|     | No. 6                   | 2,237  | 3,085        | 129,195|
| 3   | C3H/HeJ                 |        |              |      |       |
|     | No. 1                   | 823    | 27,062       |       |
|     | No. 2                   | 371    | 20,982       |       |
|     | No. 3                   | 215    | 7,151        |       |
|     | CBA/N                   |        |              |      |       |
|     | No. 1                   | 98     | 33,214       |       |
|     | No. 2                   | 82     | 18,977       |       |
|     | No. 3                   | 99     | 12,117       |       |
|     | C3J.xid                 |        |              |      |       |
|     | No. 1                   | 127    | 925          |       |
|     | No. 2                   | 123    | 1,348        |       |
|     | No. 3                   | 227    | 2,794        |       |

Spleen cells from C3H/HeN, C3H/HeJ, CBA/N, C3J.xid, and C3.CBA/N were cultured at 5 x 10^5 cells/well in modified Mishell-Dutton medium with 50 µg/ml of LPS, 10 µg/ml of NWSM, and 2 µg/ml of Con A for 3 d. 18 h before harvest, cells were pulsed with 1.0 Ci of [³H]-thymidine. Results represent arithmetic mean ± SE of triplicate wells. In experiment 1, cells were pooled from groups of three mice. In experiments 2 and 3, cells from individual mice were cultured.

had suggested that NWSM could induce an increase in incorporation of [³H]-thymidine in populations of Ig^- RTLA-Fc receptor (FcR)^+ cells (14).

Soluble anti-IgM and anti-IgD antibodies do not stimulate proliferation in spleen cells from mice with the xid defect (15). However, we have recently shown that anti-Ig antibodies conjugated to Sepharose beads can stimulate significant proliferation in cells from such mice (16). In marked contrast, however, spleen cells from C3.CBA/N mice are only marginally stimulated in response to anti-IgM or anti-IgD conjugated to Sepharose beads (Table IV).

Inability of T Cell-derived Factors to Enhance Responsiveness of C3.CBA/N Spleen Cells to TNP-BA. We have previously shown that in vitro PFC responses to the type 1 antigen TNP-BA can be enhanced by the addition of various T cell-derived lymphokines, including factors found in supernatants derived from PMA-
TABLE IV

| Exp No. | Cell source | Medium | Antiferritin bead | Anti-# bead | Anti-6 bead | LPS | Con A |
|---------|-------------|--------|-------------------|-------------|-------------|-----|-------|
| 1       | C3H/HeN     | 3.959 ± 425 | 4.965 ± 180 | 25.246 ± 1.692 | 48.164 ± 3.765 | 59.968 ± 1.277 |
|         | CBA/N       | 6.732 ± 22 | 8.734 ± 930 | 23.939 ± 1.515 | 46.035 ± 1.547 | 22.262 ± 2.672 |
|         | C3.CBA/N    | 2.210 ± 180 | 2.229 ± 82 | 3.482 ± 52 | 3.047 ± 51 | 3.743 ± 60 |
| 2       | C3H/HeN     | 2.164 ± 138 | 2.946 ± 192 | 21.605 ± 892 | ND | 25.223 ± 2.999 | 96.648 ± 5.034 |
|         | CBA/N       | 7.459 ± 369 | 10.491 ± 1.079 | 43.107 ± 2.887 | 52.884 ± 4.505 | 54.616 ± 7.91 |
|         | C3.CBA/N    | 3.192 ± 267 | 4.534 ± 569 | 9.539 ± 892 | 10.092 ± 478 | 7.211 ± 452 | 85.964 ± 4.975 |

5 x 10⁶ spleen cells from C3H/HeN, CBA/N, or C3.CBA/N were cultured in flat-bottomed microtiter wells in modified Mishell-Dutton medium. The concentration of B cell stimulators used was: LPS, 50 μg/ml; anti-lg beads at a 1:20 dilution of stock material; Con A at 2 μg/ml. After 2 d of culture, 1.0 Ci of [3H]thymidine was added and cells were harvested 18 h later. Results represent arithmetic mean ± SE of triplicate wells.

Figure 1.

Impaired Proliferative Response of C3.CBA/N Spleen Cells to Anti-lg Conjugated to Sepharose Beads

5 x 10⁶ spleen cells from C3H/HeN, CBA/N, or C3.CBA/N were cultured in flat-bottomed microtiter wells in modified Mishell-Dutton medium. The concentration of B cell stimulators used was: LPS, 50 μg/ml; anti-lg beads at a 1:20 dilution of stock material; Con A at 2 μg/ml. After 2 d of culture, 1.0 Ci of [3H]thymidine was added and cells were harvested 18 h later. Results represent arithmetic mean ± SE of triplicate wells.

Flow Microfluorometric Analysis of slg⁺ Cells in the Spleen, Lymph Node, and Peyer's Patch of C3.CBA/N mice. To examine whether the reduced responsiveness of spleen cells obtained from C3.CBA/N mice reflected a reduced number of slg⁺ cells in their lymphoid organs we stained their cells with FITC-conjugated anti-μ, anti-δ, or anti-Ia antibodies and enumerated the percentage of positively stained cells using the FACS (Table VI). Their spleens had on the average 30% fewer slgM⁺ cells as compared with CBA/N mice, and 60% fewer than C3H
### Table V

**Inability of C3.CBA/N B Cells to Respond to T Cell-derived Factors in EL4 Supernatant**

| Exp. No. | Source of cells | Medium | TNP-BA | TNP-BA + EL4-supernatant | EL4 |
|----------|-----------------|--------|--------|--------------------------|-----|
| 1        | CBA/N           | 0      | 142 (1.08) | 814 (1.16) | 24 (1.18) |
|          | C3.CBA/N        | 0      | 0 (1.12)  | 12 (1.26)  | 0   |
| 2        | C3H/HeN         | 16 (1.49) | 148 (1.25) | 746 (1.03) | 36 (1.52) |
|          | CBA/N           | 4 (1.44)  | 28 (1.42)  | 672 (1.38) | 20 (1.33) |
|          | C3.CBA/N        | 4 (1.26)  | 14 (1.48)  | 34 (1.42)  | 0   |

10⁶ spleen cells from CBA/N, C3.CBA/N, or C3H mice were cultured with TNP-BA in the presence or absence of 10% EL4 supernatant material. 3 d later, cells were harvested and anti-TNP PFC determined. Results represent geometric mean (x/± mean SE) of triplicate cultures.

### Table VI

**FACS Analysis of sIgM⁺, sIgD⁺, sla⁺, and Thy-1.2⁺ Cells Obtained from C3.CBA/N or Control Mice**

| Cell source      | Percent of positive cells |
|------------------|---------------------------|
|                  | slgM⁺ | slgD⁺ | sla⁺ | Thy-1.2⁺ |
| Spleen C3H/HeN   | 51.9  | 43.2  | 55.2 | 40.5  |
| CBA/N            | 35.4  | 27.5  | 39.3 | 57.4  |
| C3.CBA/N         | 21.9  | 11.5  | 27.5 | 32.5  |
| C3.CBA/N         | 23.2  | 11.9  | 27.6 | 32.9  |
| Lymph node       |       |       |      |        |
| C3H/HeN          | 20.9  | 23.5  | 26.8 | 87.5  |
| CBA/N            | 14.4  | 13.6  | 19.2 | 87.3  |
| C3.CBA/N         | 2.7   | 2.8   | 5.5  | 84.5  |
| C3.CBA/N         | 4.0   | 3.6   | 7.4  | 87.6  |
| Peyer's patch    |       |       |      |        |
| C3H/HeN          | 30.4  | 34.6  | 64.7 | 40.4  |
| CBA/N            | 27.5  | 24.8  | 42.9 | 61.6  |
| C3.CBA/N         | 18.0  | 13.8  | 15.8 | 42.1  |
| C3.CBA/N         | 16.7  | 14.1  | 41.0 | 44.4  |

Cells were obtained from spleen, popliteal lymph node, or Peyer's patch and stained with FITC-conjugated antibodies and analyzed on the FACS as described in Materials and Methods. Cells from two individual mice were pooled in each group.

mice; their Peyer's patch had 40% fewer slgM⁺ cells than either parental strain, and their lymph nodes had 80–90% fewer IgM⁺ cells than the control strains. An additional striking difference was noted in the increase in the numbers of slgM⁺ spleen cells that lacked detectable slgD. On the average, 75–80% of slgM⁺ splenic B cells from CBA/N mice express slgD, whereas, of slgM⁺ splenic cells from C3.CBA/N, only 40–50% express slgD and the median fluorescence intensity of these slgD⁺ cells is significantly lower than that of control cells (data not shown); the slgM⁺ cells obtained from Peyer's patches or lymph nodes from C3.CBA/N mice were of a more mature phenotype and expressed slgD as well. The fact that the numbers of Thy-1.2⁺ cells in the spleens of C3.CBA/N mice was not increased above that found in the spleens of control mice reflected the
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Table VII

In Vivo Response of C3.CBA/N to TNP-BA and TNP-Ficoll

| Cell source | Antigen  | Direct PFC/10^6 Day 5 | Direct PFC/10^6 Day 7 | Facilitated PFC/10^6 Day 5 | Facilitated PFC/10^6 Day 7 |
|-------------|----------|-----------------------|-----------------------|---------------------------|---------------------------|
|            | TNP-BA   | 148 (1.14)            | 40 (1.28)             | 26 (1.12)                 | 22 (1.74)                 |
| C3H/HeN    | TNP-BA   | 187 (1.18)            | 137 (1.08)            | 84 (1.02)                 | 53 (1.03)                 |
| CBA/N      | TNP-BA   | 71 (1.42)             | 29 (1.22)             | 10 (2.29)                 | 11 (1.46)                 |
| C3.CBA/N   | TNP-BA   | 148 (1.14)            | 40 (1.28)             | 26 (1.12)                 | 22 (1.74)                 |
| C3H/HeN    | TNP-Ficoll* | 485                  | 225                   | 229                      | 553                      |
| CBA/N      | TNP-Ficoll | 10                   | 10                    | 10                       | 10                       |
| C3.CBA/N   | TNP-Ficoll | 10                   | 10                    | 10                       | 10                       |

Mice were immunized (four per group) intravenously with 0.1 ml of TNP-BA or 50 g of TNP-Ficoll. 5 or 7 d later, spleen cells of individual mice were evaluated for numbers of direct or facilitated anti-TNP PFC.

*For evaluation of PFC responses to TNP-Ficoll, a pool of spleen cells from two C3H/HeN mice was used.

Table VIII

Reciprocal of IgM Anti-TNP Titer in Serum Obtained from Mice Injected with TNP-BA

| Mouse strain | IgM anti-TNP antibody titer Day 5 | IgM anti-TNP antibody titer Day 7 |
|--------------|----------------------------------|----------------------------------|
| C3.CBA/N     | 22,115 (1.17)                    | 17,552 (1.22)                    |
| CBA/N        | 24,852 (1.44)                    | 25,399 (1.61)                    |
| C3H/HeN      | 108,664 (1.32)                   | 90,905 (1.15)                    |

Mice (three per group) were immunized intravenously with 0.1 ml of a 1:10 dilution of stock TNP-BA and sera were collected 5 and 7 d later. Preimmune anti-TNP titers were 204 (1.85) and 1,102 (2.90) for C3.CBA/N and CBA/N mice, respectively.

Presence of an increase in the percentage of Thy-1.2^- slg^- cells in these mice.

Responsiveness of C3.CBA/N Mice to In Vivo Challenge with TNP-BA. To investigate the immune potential of C3.CBA/N mice to in vivo challenge with antigens, we immunized them with type 1 antigen TNP-BA and type 2 antigen TNP-Ficoll (Table VII). As expected, the mice with the xid defect were unresponsive to TNP-Ficoll; however, all three strains responded to immunization with TNP-BA. The facilitated PFC response of both CBA/N and C3.CBA/N was lower than that of C3H mice. In another experiment, C3H, C3.CBA/N, and CBA/N mice were immunized intravenously with TNP-BA, and serum titers of anti-TNP antibody were determined (Table VIII). No significant difference was observed in the IgM anti-TNP antibody response between the CBA/N and C3.CBA/N.

Antibody-dependent Cell-mediated Cytotoxicity of Spleen Cells from C3J.xid Mice. The level of antibody-dependent cell-mediated cytotoxicity that reflects the activity of the Ig^- Thy-1.2^- spleen cells was compared in spleen cells from C3J.xid and parental strain mice. Spleen cells were cultured (10^6, 2.5 × 10^6, and 5 × 10^6) in the presence of 10^5 51Cr-labeled CE at a dilution of 1:3,200 rabbit anti-CE. At all effector/target cell ratios tested, there was a significant increase of specific
Lack of Complementation of the B Cell Immune Defect in an F1 Female Mouse Obtained from the Mating of C3J.xid and C3.CBA/N

### Table IX

| Strain              | Anti-TNP PFC response to TNP-BA | Proliferative response to: |
|---------------------|---------------------------------|---------------------------|
|                     |                                  | Medium | LPS         |
|                     | Response to TNP-BA               |        |            |
| C3H/HeN             | 438 (1.03)                      | 2,045 ± 236 | 49,228 ± 3,746 |
| CBA/N               | 428 (1.15)                      | 824 ± 32  | 38,149 ± 3,683 |
| C3J.xid             | 94 (1.17)                       | 2,177 ± 222 | 3,493 ± 468  |
| (C3J.xid × C3.CBA/N)F1 | (C3J.xid × C3.CBA/N)F1         |        |            |
| No. 1               | 48 (1.05)                       | 1,368 ± 147 | 4,083 ± 2,720 |
| No. 2               | 54 (1.09)                       | 2,083 ± 270 | 5,142 ± 3,059 |
| No. 3               | 0                              | 2,685 ± 91  | 6,293 ± 3,608 |

Spleen cells from mice were cultured at 10^6/well for evaluation of in vitro anti-TNP PFC responses to TNP-BA, and harvested 3 d later. For evaluation of the proliferative response to LPS (50 µg/ml), cells were cultured at 5 × 10^5/well and harvested on day 5 of culture, following an 18-h pulse with [3H]thymidine. In data not presented, culturing of these cells at 2.5 × 10^5/well or harvesting 1 d earlier did not show any significant difference from that reported in the table. Results of proliferative responses to LPS and medium represent the arithmetic mean ± SE of triplicate wells. Pools of two CBA/N, C3J.xid, and C3H/HeN were used. Results for anti-TNP PFC represent geometric mean (x/± mean SE).

51Cr release when cells from C3J.xid mice were used as compared with cells from C3H/HeN or CBA/N strains (Fig. 2). These results are consistent with the increased numbers of Thy-1.2− sIg− cells found in these mice.

Lack of Complementation of the Immune Defects in an F1 Female Mouse Resulting
from the Mating of C3.CBA/N and C3J.xid. To determine whether the genetic basis for the immune defects of the C3.CBA/N and C3J.xid were similar, female progeny of a cross of these two strains were studied (Table IX). Both the in vitro proliferative response stimulated by LPS and the in vitro antibody response to TNP-BA was impaired in the cells from these mice, suggesting that the immune defects of these two strains of mice reflect a common genetic derangement. It should be noted, however, that since individual cells express only a single X chromosome, F₁ females should actually be chimeras of C3.CBA/N and C3J.xid B cells. The fact that such F₁ females have a defect similar to both parents suggests that the functions of their B cells do not complement one another. The result does not formally demonstrate that the genetic defects are identical. However, since both strains are derived by placing the CBA/N X chromosome (xid) on a C3H background, it seems likely that their defects would be the same.

Discussion

The introduction of the xid gene onto the C3H background leads to a profound B cell immunodeficiency characterized by in vitro B lymphocyte unresponsiveness to both type 1 and 2 antigens as well as to a series of B cell mitogens including LPS, NWSM, and anti-Ig antibody conjugated to Sepharose beads. These defects appear to be intrinsic to B cells since (a) the addition of C3H/HeN T cells and macrophages to C3.CBA/N spleen cells does not allow a response to occur, and (b) spleen cells from C3.CBA/N mice do not suppress antibody responses of C3H/HeN spleen cells. Our results showing that the introduction of xid into either C3H/HeN or C3H/HeJ mice leads to comparable deficits indicates that the LPS$^a$ gene is not critical for this synergistic defect. Furthermore, introduction of the xid gene into a different LPS$^a$-bearing strain, C57BL10/ScN, does not result in unresponsiveness to either TNP-BA or to Boivin preparations of LPS (unpublished observations).

Mice of the C3.CBA/N strain do mount in vivo anti-TNP responses to TNP-BA that are comparable in magnitude to that of CBA/N mice, and in recent studies we have shown that C3J.xid mice mount normal primary and secondary in vivo antibody responses to TNP-KLH and to the hemagglutinin of PR8 influenza virus (unpublished observations). Thus, the synergistic defect, although profound, does not preclude antibody responsiveness in general.

The in vitro unresponsiveness of these mice obviously does not reflect the absence of B cells. Although this is implicit in their ability to make in vivo responses to TNP-BA and PR8, it is directly established by studies of their B cells. The percent of sIgM$^+$ cells in spleens and Peyer's patches of C3.CBA/N mice is ~30% less than that of CBA/N mice and ~60% less than that of C3H/HeN mice; a greater decrease in the percent of sIgM$^+$ cells was observed in the peripheral lymph nodes of these immune-defective mice. It is of particular interest that the frequency of sIgD$^+$ cells in the spleen of C3.CBA/N mice is markedly reduced, to 50% of the number of sIgM$^+$ cells. The high frequency of sIgM$^+$ sIgD$^-$ cells in these mice could reflect either the relatively large numbers of immature B cells or the presence of activated B cells that have lost sIgD. Since lymph nodes normally contain large numbers of activated B lymphocytes but have few immature B lymphocytes, the paucity of sIg$^+$ cells in lymph nodes from
C3.CBA/N mice suggests that the sIgM⁺ sIgD⁻ spleen cells represent a largely immature rather than an activated population of B cells.

The inability of cells from C3.CBA/N mice to respond in vitro to type 1 antigens or to B cell mitogens may be a result of their inability to respond to various lymphokines that influence B cell growth, such as those present in EL4 supernatant. This suggestion is premised on the idea that for in vitro responses to type 1 and 2 antigens and to B cell mitogens, including insolubilized anti-Ig, it is not antigen-specific T cells that influence the magnitude of the response but rather T cell-derived lymphokines that are required to enhance B cell responsiveness. *B. abortus* functions in vitro as a type 1 antigen, but it also has been shown to stimulate antigen-specific T cells in vivo (18). Thus, it is not surprising that in vivo immunization of normal mice with TNP-BA can stimulate responses dependent on carrier-specific T cells as well as factor-dependent responses. Our observation that C3.CBA/N and C3J.xid mice respond in vivo to the T cell-dependant antigen TNP-KLH suggests that the pathway dependent on carrier-specific T cells is intact in these animals. We propose that the in vivo response of C3.CBA/N mice to TNP-BA results from the action of carrier-specific T cells. Based on these observations, we propose the following model (Fig. 3) to explain the "synergistic defect" of C3.CBA/N and C3J.xid mice: C3H/HeN and C3H/HeJ mice may have an immune defect in B cell function that is not normally detected but which in an xid mouse has a profound effect on the overall B cell response. Such mice with the xid defect lack a mature subset of Lyb5⁺ B cells and have only Lyb5⁻ B cells that can be activated by antigens via two alternative routes, that involving carrier-specific T cells and that involving type 1 antigens (e.g., TNP-BA, LPS) and soluble factors. In vitro, where carrier-specific T cell help is limiting, stimulation of resting Lyb5⁻ B cells with a combination of type 1 antigen and lymphokines leads to B cell proliferation and differentiation. Since B cells from C3.CBA/N mice, however, are poorly responsive to type 1 antigens and to factors in EL4 supernatant, it would be reasonable to propose that the defect of these mice lies in the insensitivity of their Lyb5⁻ B cells to stimulation along that pathway of activation that is available to "normal" Lyb5⁻ cells.

**Figure 3.**

The pathway of factor-dependent activation of resting Lyb5⁻ cells by type 1 antigens cannot be initiated by T cell-dependent antigens in a primary in vitro antibody response since responsiveness to this class of antigens requires the participation of a number of T cell-derived lymphokines to which Lyb5⁻ B cells from CBA/N mice are unable to respond (19, 20). The second pathway of activation that is available to Lyb5⁻ B cells proceeds via interaction with a carrier-specific, major histocompatibility complex (MHC)-restricted T helper cell. This mode of activation stimulates Lyb5⁻ cells from CBA/N mice as well as from
C3.CBA/N and C3J.xid. Thus, in vivo, where carrier-specific T cells are in less limiting numbers than in vitro, B cells of C3.CBA/N mice can be stimulated to antibody production by either T cell-dependent antigens or by a number of type 1 antigens that can also function as T cell-dependent antigens and thus are able to stimulate carrier-specific T cells. The C3J.xid and C3.CBA/N strains of mice therefore appear to be entirely limited in their B cell responses to the use of cognate interaction involving the interactions of carrier-specific T cells with B cells. This should provide an excellent model for the examination of such B cell activation in in vivo and in vitro immune responses.

Summary

Introduction of the CBA/N X-linked gene into C3H mice has resulted in the establishment of a new strain of mice that has profound immunologic defects. B cells from these mice show significantly impaired in vitro immune responses to the T cell-independent type 1 antigen trinitrophenyl-Brucella abortus (TNP-BA) as well as markedly reduced proliferative responses to a number of B cell mitogens when compared with the responses of the parental control mice. The in vivo response of such mice to TNP-BA is, however, comparable to that of CBA/N mice. Furthermore, B cells from C3.CBA/N mice are unresponsive to the plaque-forming cell enhancing effects induced by EL4-derived supernatant in the presence of TNP-BA, unlike B cells obtained from CBA/N or C3H/Hen mice whose responsiveness to TNP-BA can be significantly enhanced in the presence of EL4-derived supernatant. The model we have presented to best explain these results suggests that B cells from C3.CBA/N mice can be stimulated only under conditions in which they can interact with carrier-specific T cell help and not under conditions where factor-dependent responses are dominant.

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