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*

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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)F_1 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*^{d}-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),[1] Nocardia water-soluble mitogen (NWSM), and antiimmunoglobulin (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. F_1 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). F_1 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-aminoethylcarbamyl-methyl-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

[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; sIg, 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 1.6×10^{-4}. CE target cells (10^7) were incubated for 1 h at 37°C with 150 μCi of sodium-^{51}chromate (^{51}Cr) (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. 2.5×10^6 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 ^{51}Cr-labeled CE in the presence of normal rabbit serum at the same dilution with ^{51}Cr-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 = $[(cpm_{Ab} - cpm_{SR})/(cpm_T - cpm_{SR})] \times 100$, where cpm_{Ab} is the counts in supernatant of the tubes with rabbit serum (normal or anti-CE), cpm_{SR} is the spontaneous release of counts in the control tubes with nonimmune serum, and cpm_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 Clorborn 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 (5×10^{-4} μg/ml) or TNP-BA (10^{-3} 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 5×10^5 or 2.5×10^5 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/mmole) 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 sIg$^+$ 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

TABLE I

In Vitro Response of Spleen Cells Obtained from C3.xid or C3.CBA/N to TNP-BA

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	
	No. 1	20 (1.05)
	No. 2	40 (1.11)
	No. 3	0
	No. 4	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*	
	No. 1	0
	No. 2	0
	No. 3	0
	No. 4	0

10^6 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, 5×10^5, or 4×10^5 cells/well in experiments 1–3, respectively, together with 5×10^5 C3J.*xid* cells and TNP-BA. 3 d later anti-TNP PFC were determined. Results represent the geometric mean (x/÷ 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 2.5×10^5 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 [^{3}H]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

In Vitro Proliferative Response of C3.CBA/N and C3J.xid to B and T Cell Mitogens

Exp.	Strain (number of mice)	Medium	LPS (Boivin)	NWSM	Con A
			cpm		
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	1,381	3,716		65,581
	No. 4	2,799	4,319		137,478
	No. 5	1,636	3,432		141,295
	No. 6	2,237	3,085		129,195
3	C3H/HeJ				
	No. 1	823		27,062	
	No. 2	371		20,932	
	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 5×10^5 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 [^{3}H]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 [^{3}H]-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-

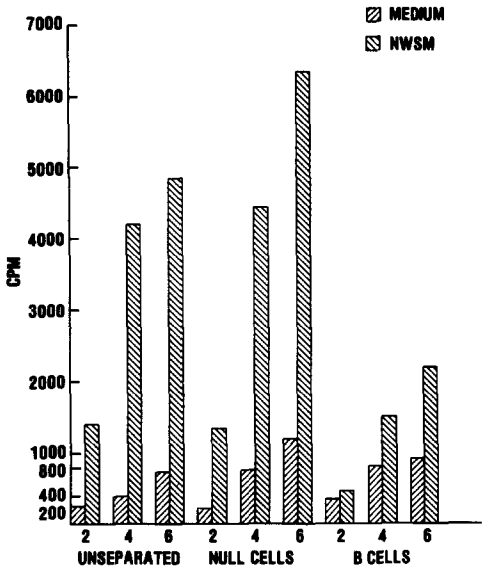

FIGURE 1.

TABLE IV

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

Exp. No.	Cell source	Medium	Antiferritin bead	Anti-μ bead	Anti-δ bead	LPS	Con A
				cpm			
1	C3H/HeN	3,959 ± 425	4,965 ± 180	23,246 ± 1,892	48,164 ± 3,763	30,908 ± 1,277	
	CBA/N	6,732 ± 92	8,734 ± 930	25,959 ± 1,515	46,035 ± 1,587	22,262 ± 2,672	
	C3.CBA/N	2,210 ± 180	2,229 ± 82	3,482 ± 52	3,047 ± 511	3,743 ± 601	
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 ± 569	10,491 ± 1,079	43,107 ± 2,887	52,884 ± 4,505	54,616 ± 791	85,755 ± 7,096
	C3.CBA/N	3,192 ± 267	4,534 ± 369	9,959 ± 892	10,002 ± 478	7,211 ± 452	85,964 ± 4,375

5×10^5 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-Ig beads at a 1:20 dilution of stock material; Con A at 2 μg/ml. After 2 d of culture, 1.0 Ci of [^{3}H]thymidine was added and cells were harvested 18 h later. Results represent arithmetic mean ± SE of triplicate wells.

stimulated EL4 thymoma cell line (17). To determine whether the impaired responses of C3.CBA/N to TNP-BA could be enhanced by soluble factors in such supernatants, we cultured their splenic B cells with TNP-BA and EL4-derived supernatant (Table V). Although this factor markedly enhanced PFC responsiveness of cells from CBA/N and C3H/HeN mice to type 1 antigens, it had little or no effect on C3.CBA/N cells.

Flow Microfluorometric Analysis of sIg$^+$ 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 sIg$^+$ 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 sIgM$^+$ 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	Anti-TNP PFC/culture			
		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

10^6 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$^+$, sIa$^+$, and Thy-1.2$^+$ Cells Obtained from C3.CBA/N or from Control Mice

Cell source		Percent of positive cells			
		sIgM$^+$	sIgD$^+$	sIa$^+$	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	5.3	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 sIgM$^+$ 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 sIgM$^+$ spleen cells that lacked detectable sIgD. On the average, 75–80% of sIgM$^+$ splenic B cells from CBA/N mice express sIgD, whereas, of sIgM$^+$ splenic cells from C3.CBA/N, only 40–50% express sIgD and the median fluorescence intensity of these sIgD$^+$ cells is significantly lower than that of control cells (data not shown); the sIgM$^+$ cells obtained from Peyer's patches or lymph nodes from C3.CBA/N mice were of a more mature phenotype and expressed sIgD 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

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

Cell source	Antigen	Direct PFC/10^6		Facilitated PFC/10^6	
		Day 5	Day 7	Day 5	Day 7
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	533
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	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)	90,903 (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$^-$ sIg$^-$ 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 Cytoxicity 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 5×10^5) in the presence of 10^5 ^{51}Cr-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

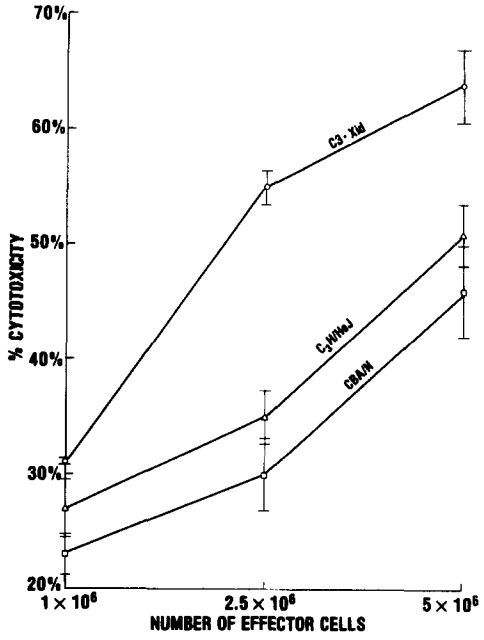

FIGURE 2.

TABLE IX

Lack of Complementation of the B Cell Immune Defect in an F_1 Female Mouse Obtained from the Mating of C3J.xid and C3.CBA/N

Strain	Anti-TNP PFC response to TNP-BA	Proliferative response to:	
		Medium	LPS
		cpm	
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)F_1			
No. 1	48 (1.05)	1,363 ± 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 3 of culture, following an 18-h pulse with [^{3}H]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).

^{51}Cr 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 F_1 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, F_1 females should actually be chimeras of C3.CBA/N and C3J.*xid* B cells. The fact that such F_1 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^d gene is not critical for this synergistic defect. Furthermore, introduction of the *xid* gene into a different LPS^d-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.

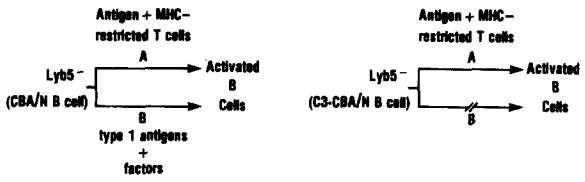

FIGURE 3.

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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