

## LACK OF MATURE B CELLS IN NUDE MICE WITH X-LINKED IMMUNE DEFICIENCY\*

By HENRY H. WORTIS, LINDA BURKLY, DEBBIE HUGHES,  
SANDRA ROSCHELLE, AND GERRY WANECK

*From the Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111*

The normal B cell differentiation pathway is not known in detail. In the mouse, one mutation (*x-linked immune deficiency, xid*)<sup>1</sup> blocks the differentiation of late-maturing B cells expressing the cell surface antigens Lyb<sub>3</sub> (1), Lyb<sub>5</sub> (2), Lyb<sub>7</sub> (3), and M1s determinants (4). In H-2<sup>b</sup> mice, this population expresses the Ia antigen IaW39 (5). These cells are responsible for the entire response to type 2 thymus-independent antigens such as trinitrophenyl (TNP)-Ficoll (2, 6, 7). It has not been established whether these cells are a late stage of antigen-independent differentiation of all normal B cells or a subpopulation resulting from an early branch point in B cell differentiation. The absence of in vitro colony-forming B cells in fetal *xid* mice (8) suggests that *xid* blocks the early differentiation of a B cell subset.

There are some observations that suggest a defect in B cell development in nude mice. The electrophoretic mobility of nude and normal B cells differ (9); both nude and normal B cells bind to macrophages, but only normal B cells are released by the addition of T cells (M. O'Toole and H. H. Wortis, unpublished observation), and nude B cells fail to respond to T cell help in lipopolysaccharide (LPS)-generated polyclonal responses (10). Although these observations are hardly compelling, it is worth noting that *xid* mice produce low amounts of IgM and IgG<sub>3</sub> and normal amounts of IgG<sub>1</sub>, IgG<sub>2b</sub>, IgG<sub>2a</sub>, and IgA (11), whereas nudes produce normal amounts of IgM but little IgG<sub>1</sub>, IgG<sub>2b</sub>, IgG<sub>2a</sub>, and IgA (reviewed in 12). Furthermore, in *xid* mice, the ratio of  $\kappa$  to  $\lambda_1$  serum immunoglobulins (Ig) is unusually high, whereas in nude mice this ratio is abnormally low.<sup>2</sup> Nudes make poor responses to thymus-dependent antigens and normal responses to thymus independent antigens, whereas in *xid* mice the reverse is true. In other words, phenotypically *nude* and *xid* resemble complementary defects of antibody production. We felt it possible that *nude* and *xid* represented two independent mutations that affected two limbs of normal B cell development. To test this hypothesis, we bred CBA nude mice with the *xid* defect. If *nude* and *xid* each control one limb of normal B cell development, then we thought doubly defective mice would lack normal B cells. Studies to test this hypothesis are reported in this paper.

\* Supported in part by grants AI-15803-02 and P30-23776-01 from The National Institutes of Health.

<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; Con A, concanavalin A; FITC, fluorescein isothiocyanate; 2ME, 2-mercaptoethanol; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TI-1, thymus-independent antigen type 1; TI-2, thymus-independent antigen type 2; TNP-trinitrophenyl; *xid*, x-linked immune deficiency.

<sup>2</sup> Burkly, L., and H. H. Wortis. Influences of the *nude* and *x-linked immune deficiency* genes on  $\kappa$  and  $\lambda_1$  expression. Manuscript in preparation.

### Materials and Methods

*Mice.* CBA/Tufts and CBA/Tufts nude (13) mice are maintained as inbred stocks at Tufts University School of Medicine, Boston, MA. CBA/N mice are bred at Tufts from a nucleus supplied by Dr. Harvey Cantor of Sidney Farber Cancer Center, Boston, MA.  $nu/+ X^{xid}X^+F_1$  females were from an original CBA/N  $\times$  CBA/Tufts. Nude ( $N_{12}F_8$ )  $nu/nu$  cross were mated to CBA/Tufts  $nu/nu$  males. The nude offspring were phenotyped by testing for responses to TNP-Ficoll.  $nu/+ X^{xid}Y$  mice were backcrossed again to CBA/Tufts females. The doubly heterozygous females were backcrossed again to nude males, and the cycle was repeated. We are now at the 7th backcross generation and have begun brother-sister matings.

Specific pathogen-free stocks are maintained in a sealed room in which incoming air is HEPA filtered. The room is kept under positive air pressure. Temperature (26°C) and humidity (40%) are controlled automatically. The room is on a 13-h, 11-h light/dark cycle. It is washed down regularly with bactericidal Bactophene (Oxford Chemical Co., Winthrop, MA). Only two workers entered the room. The mice were housed in standard polycarbonate cages with a specially manufactured tight-fitting cover containing a bacterial filter (spun filtered polyester, 2024; Dupont Chemical, Wilmington, DE). The room contained a Baker laminar flow hood (EGB3252; Baker Co., Inc, Sanford, ME) in which all animal handling, including cage changing, was done. The mice were handled with sterile gloves or with forceps dipped in Bactophene between cages. Bedding, food, and water were autoclaved before use.

Cultures were taken from random cages at regular intervals and from suspect mice when necessary. Selected non-nude mice were bled at quarterly intervals and tested for antibody to a panel of viruses. No bacterial pathogens were identified in the intestinal flora. No mice with antibodies to the common viral pathogens were found in the colony.

*Antigens.* Haptenated derivatives of Ficoll and *Escherichia coli* O127:B8 LPS and *Brucella abortus* were prepared as previously published (14) and generously contributed by Dr. Peter Brodeur, Institute for Cancer Research, Philadelphia, PA.

*Phenotyping.* Male offspring of test crosses ( $nu/+ X^{xid}/X^+ \times nu/nu X^+/Y$ ) were immunized intraperitoneally with 10  $\mu$ g of fluorescein isothiocyanate (FITC)-Ficoll or TNP-Ficoll in saline when they were 6–8 wk of age. 5 d later, they were tail bled, and anti-TNP was antibody measured by hemagglutination, using TNP-sheep erythrocytes (TNP-SRBC). Mice that failed to produce antibody to this TI-2 antigen were considered to bear the *xid* defect.

*Isotype-specific Sera.* BALB/c myelomas were obtained from Dr. M. Potter. From ascites, we then prepared purified proteins. HOPC-1 ( $\gamma_{2a}\lambda_1$ ), MPC-11 ( $\gamma_{2b}\kappa$ ), and MOPC21 ( $\gamma_1\kappa$ ) were purified by ammonium sulfate precipitation, Sephadex G200 gel filtration, and chromatography on DEAE-cellulose. J606 ( $\gamma_3\kappa$ ) was precipitated at pH 8 in low ionic strength buffer (15). MOPC 104E ( $\mu\lambda_1$ ) and MOPC 460 ( $\alpha, \kappa$ ) were affinity purified on  $\alpha$ 1,3 dextran-bovine serum albumin (BSA)-Sepharose and dinitrophenyl-lys-Sepharose, respectively. Purified proteins were conjugated to cyanogen bromide-activated Sepharose 4B and used as immunoadsorbants.

Goats and/or rabbits were immunized with 100  $\mu$ g purified protein in complete Freund's adjuvant. They were boosted at monthly intervals and bled 1 and 2 wk later. The resultant antibody was then purified by ammonium sulfate precipitation and both negative and positive adsorption and elution on affinity columns of the appropriate myeloma proteins. Only antibody with <5% binding to heterologous isotypes, as determined by a radioimmunoassay (13), is considered pure.

Antisera specific for  $\lambda_1$  light chains were prepared from goat antisera to MOPC 104E by positive adsorption onto a HOPC-1 Sepharose column. Bound material was eluted with 0.1 M glycine HCl, pH 2.3, and rendered highly specific by passage over SJL normal serum Sepharose (SJL having little to no  $\lambda_1$  protein) (16). Antisera specific for  $\kappa$  light chains was prepared from rabbit and goat antisera to mouse Fab fragments. After adsorption onto a J606 Sepharose column, acid-eluted material was successively passed over HOPC-1 Sepharose and MOPC 104E Sepharose to remove any residual anti- $\lambda_1$  activity.

*Serum  $\kappa$  and  $\lambda_1$  Levels.* Briefly, levels of  $\lambda_1$ - and  $\kappa$ -bearing Ig were determined by competition radioimmunoassays using purified HOPC-1 ( $\gamma_{2a}\lambda_1$ ) or MOPC-460 ( $\alpha, \kappa$ ) as insoluble antigens and standards together with  $I^{125}$ -labeled anti- $\lambda_1$  or anti- $\kappa$ . In some instances, serum Ig levels were determined by essentially comparable methods by Jordan Krieger, working in the

laboratory of Dr. Herman Eisen, Massachusetts Institute of Technology, Cambridge, MA (see Results).

*Cell Preparations.* Suspensions of thymus, lymph node, and spleen cells were made by gentle mincing through a 100 mesh wire screen. Erythrocytes were removed by brief treatment with Tris  $\text{NH}_4\text{Cl}$  with 0.4% BSA (17). Dead cells were removed by incubation with low ionic strength buffer and passage through a cotton plug (18). Viability was assessed by eosin dye exclusion.

*Fluorescence.* Goat antibodies specific for  $\mu$ ,  $\kappa$ , and  $\lambda_1$  were coupled with biotinyl-*N*-hydroxy-succinimide ester (19). Fluorescein-conjugated avidin (Becton, Dickinson & Co., Sunnyvale, CA) is then added as a second step reagent.

Cell suspensions free of erythrocytes and dead cells were fixed in paraformaldehyde (20) to prevent capping during staining and to permit samples to remain stable.  $5 \times 10^5$  fixed cells were incubated at room temperature for 30 min with 50  $\mu\text{l}$  of an appropriate dilution of biotin-conjugated antibody. After three washes in 2% normal goat serum-phosphate-buffered saline, the cells were incubated for 30 min with FITC-Avidin. After three washes, the cells were resuspended and examined with a fluorescence microscope with epi-illumination.

*Mitogenic Responses.*  $5 \times 10^5$  spleen cells were cultured in 96-well flat bottomed microtiter plates (Microtest II, 3040; Falcon Labware, Oxnard, CA) in 200  $\mu\text{l}$  of medium RPMI with 5% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 20 mM Hepes, and 2 mM glutamine. Mitogens include K-235 LPS (a generous gift from Dr. Floyd McIntyre, University of Colorado Medical Center, Denver, CO) Concanavalin A (Con A) (Difco Laboratories, Detroit, MI), dextran sulfate (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ), and O127:B8 LPS (Difco Laboratories). Some cultures contained  $5 \times 10^{-5}$  M 2-mercaptoethanol. Cultures were maintained in a 5%  $\text{CO}_2$  humidified incubator at 37°C. 48 h after the initiation of the culture, 1  $\mu\text{Ci}$  of (methyl)- $^3\text{H}$ thymidine (6.7 Ci/mm) was added. 18–24 h later, the cells were harvested, and the amount of cell-incorporated radioactivity was measured in a scintillation counter.

*Mishell-Dutton Assays.* Spleen cells ( $5 \times 10^5$ ) were cultured in microtiter plates in 0.2 ml Iscove and Melcher's (21) modified medium (78-5220; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), supplemented with 100  $\mu\text{g}/\text{ml}$  streptomycin sulfate (Gibco Laboratories) and 100 U/ml penicillin G (Pfizer Inc., New York),  $5 \times 10^{-5}$  M 2-mercaptoethanol (2ME), and 10% heat-inactivated fetal calf serum. Cultures were incubated in a humidified 37°C, 5%  $\text{CO}_2$ -air incubator for 3–4 d (depending on the antigen) and then harvested and washed in Hanks' buffered salt solutions with 0.1% BSA and 10 mM Hepes. Three to four wells were pooled as a culture group, and three such groups were examined for each treatment.

*Plaque-forming Cell Assay.* A slide modification of the Jerne plaque assay was used to measure antibody to TNP-SRBC or FITC-SRBC (14). Indirect plaques were developed with a rabbit antiserum that reacts with  $\gamma_1$ ,  $\gamma_{2a}$ , and  $\gamma_{2b}$  isotypes. Selected guinea pig complement is used to effect hemolysis.

*Histology.* Selected organs were fixed in Bouin's solution. Sections were stained with hematoxylin and eosin.

*Peripheral Lymphocyte Counts.* White blood cell counts were made using blood samples diluted in 2% acetic acid and Neubauer counting chambers. Smears of whole blood were stained with Wright's Giemsa. At least 200 leukocytes were counted per differential count.

## Results

*Serum Ig Levels.* To see whether nude, xid mice had normal levels of serum Ig, sera from individual mice were allowed to react with heavy chain isotype-specific antisera in agar double-diffusion. As shown in Table I, most doubly deficient mice had undetectable levels of all heavy chains. In contrast, nude mice had some antibody of all isotypes, and xid mice had low amounts of  $\mu$  and  $\gamma_3$ , as previously reported (11).

Because these results suggested that nude xid mice have severe hypogammaglobulinemia, we measured serum Ig levels using a quantitative radio-immunoassay for the  $\kappa$  and  $\lambda_1$  light chain isotypes. The data presented in Table II shows that doubly deficient mice have <5% of normal levels of both  $\kappa$ - and  $\lambda_1$ -bearing Ig. Measurements

TABLE I  
*Ig Isotypes in Normal and Mutant Mice*

Phenotype	Isotype					
	$\mu$	$\gamma_3$	$\gamma_1$	$\gamma_{2b}$	$\gamma_{2a}$	$\alpha$
Normal	6/6*	3/3	6/6	6/6	6/6	3/3
Nude	7/7	4/4	5/7	6/6	7/7	4/4
xid	3/9	1/6	9/9	6/6	8/9	6/6
Nude xid	2/6	0/3	0/6	0/3	1/6‡	0/3

\* Number positive on agar double diffusion per number tested.

‡  $\gamma_{2a}$ -positive nude xid was also  $\mu$  positive.

TABLE II  
*Serum Ig*

Experiment	Isotype	Phenotype			
		Normal	Nude	xid	Nude xid
				$\mu\text{g/ml}$	
1	$\kappa$	3,200	2,400	3,100	Not done
	$\lambda_1$	72	62	3.7	8.2
2	$\kappa$	3,000	2,600	4,000	46
	$\lambda_1$	72	46	18.8	1.8
3	$\kappa$	1,700	1,500	1,700	13.4
	$\lambda_1$	54	Not done	14.8	<2
4	$\kappa$				136
	$\lambda_1$				<2
	$\kappa$				40
	$\lambda_1$				1
	$\kappa$				62
	$\lambda_1$				1.5
	$\kappa$				41.5
	$\lambda_1$				0.3

The values presented were determined for individual mice.

of  $\lambda_2$ , performed in Dr. Herman Eisen's laboratory, revealed levels in doubly defective mice at 10% of normal (data not shown).

*In Vitro Responses to Antigen.* We then measured the ability of these mice to respond to TNP Brucella abortus (a nonmitogenic thymus-independent type 1 antigen) (22), TNP-LPS (a mitogenic TI-1 antigen), TNP-Ficoll (a TI-2 antigen), and LPS (a polyclonal activator). As expected, both nude and normal mice responded to all of these thymus-independent antigens (Table III). Mice with the xid defect failed to respond to TI-2 antigen or to the low dose of the polyclonal stimulator LPS. Surprisingly, the double defective mice failed to respond to either TNP-BA or TNP-LPS, two TI-1 antigens, a defect not found in either of the parental strains.

*In Vitro Responses to Mitogens.* The failure of B cells in nude xid mice to produce antibody in vivo (as reflected by the low serum Ig levels) or in vitro (seen in the Mishell-Dutton assays) could be because of a defect at any one of several levels (e.g., secretion, antigen recognition, antigen presentation, accessory cells, etc.) To see whether there were B cells that could respond by proliferating in response to mitogenic signals, a variety of B cell mitogens were used as stimulators in an in vitro assay.

TABLE III  
*In Vitro* PFC Response to a Variety of Thymus-independent Antigens

Antigen	Phenotype			
	Normal	Nude	xid	Nude xid
$\bar{o}$	93* (1.64)	51 (1.3)	6 (1.26)	<5 (1)
TNP-Ficoll	1 ng	1,093 (1.04)	811 (1.05)	31 (1.14)
	10 ng	1,294 (1.11)	772 (1.03)	<6 (1)
	100 ng	1,112 (1.04)	527 (1.14)	6 (1.26)
TNP-BA	1/100	633 (1.04)	889 (1.08)	399 (1.17)
	1/33	585 (1.13)	203 (1.04)	161 (1.09)
	1/10	902 (1.03)	149 (1.3)	156 (1.22)
TNP-LPS	10 ng	1,842 (1.08)	301 (1.3)	941 (1.11)
	100 ng	1,075 (1.08)	1,125 (1.15)	942 (1.03)
	1,000 ng	1,115 (1.12)	820 (1.16)	1,380 (1.04)
LPS	10 ng	174‡ (1.17)	56 (1.1)	14 (1.26)
	100 ng	552 (1.05)	186 (1.08)	59 (1.32)
	1,000 ng	714 (1.12)	279 (1.15)	380 (1.18)

\* Anti-TNP PFC microwell geometric mean ( $\bar{x}$  SEM).

‡ In the LPS-stimulated cultures, the PFC include both anti-TNP and anti-SRBC PFC.

TABLE IV  
 Mitogen-induced Proliferation of Mutant and Normal Spleen Cells

Experiment	Mitogen	Phenotype			
		Normal	Nude	xid	Nude xid
1	Medium	3.8* (1.02)	3.9 (1.05)	0.2 (1.27)	1.6 (1.18)
	0.33 $\mu$ g Con A	388.8 (1.00)	2.9 (1.05)	325.5 (1.05)	1.3 (1.07)
	1 $\mu$ g LPS	171.2 (1.08)	150.9 (1.12)	10.7 (1.07)	0.6 (1.12)
	<i>E. coli</i> O127:B8				
2‡	Medium	38.2 (1.07)	14.3 (1.02)	0.9 (1.04)	0.5 (1.06)
	1 $\mu$ g LPS	132.9 (1.05)	ND§	78.6 (1.01)	0.5 (1.32)
	K 235	80.5 (1.02)	35.3 (1.07)	ND	4.3 (1.05)
	10 $\mu$ g dextran sulfate				

\* Counts per minute; geometric mean  $\times 10^{-3}$  (SEM) triplicate values [ $H^3$ ]thymidine incorporation.

‡ In experiment 2, 2ME at  $5 \times 10^{-5}$  M was added to the medium.

§ Not done.

Because neither nude nor nude xid mice have mature T cells, it was expected that Con A would fail to stimulate thymidine uptake by their spleen cells. This was confirmed (Table IV). K235 LPS, a lipid A mitogen for B cells (23), triggered proliferation of normal and nude cells and less intense stimulation of xid cells (Table IV). LPS O127:B8 with both lipid A and lipoprotein mitogens stimulated normal, nude, and xid mitosis. Neither form of LPS stimulated mitosis in nude xid mice, even when used at doses of 1, 10, or 100 ng (data not shown). Again, the defect in the doubly defective mice is greater than that seen in either nude or xid mice. Neither xid nor nude xid mice responded to 2ME, as expected (24) (data not shown). All mice, including the nude xid, responded weakly to dextran sulfate (25).

TABLE V  
*Frequency of Surface Ig-positive Spleen Cells*

Experiment	Phenotype			
	Normal	Nude	xid	Nude xid
1	$\mu$ 40%	59%	35%	<1%
	$\kappa$ 36	50	39	1
	$\lambda_1$ * 5	3	7	<1
2	$\mu$ 44	48	30	13
	$\kappa$ * 43	50	31	10
	$\lambda_1$ ‡ 3	3	2	2
3	$\lambda_1$ 2	2	1	1
	$\mu$ 45	57	32	3
	$\kappa$ 53	64	41	4
	$\lambda_1$ * 2	3	2	<0.1

Frequencies of spleen cells with  $s\mu$ ,  $s\kappa$ , and  $s\lambda_1$ . Mice 8–12 wk old were used, and 200–400 cells were counted for each determination.  $s\lambda_1$  was identified with goat anti-mouse  $\lambda_1$  or SJL anti- $\lambda_1$  (LS136) (27). LS136 was generously given to us by Dr. Michael Reth and Dr. Klaus Rejewsky.

\* Using goat anti-mouse  $\lambda_1$ .

‡ Using LS136 SJL anti- $\lambda_1$ .

*Surface Ig.* Because no evidence could be found establishing the existence of functional B cells in nude xid mice, we looked for cells with surface Ig (sIg), the hallmark of mature B cells (26). The frequency of spleen cells with  $s\mu$ ,  $s\kappa$ , and  $s\lambda_1$  in mice of the four studied phenotypes is shown in Table V. The frequencies of  $\mu$ -,  $\kappa$ -, and  $\lambda_1$ -bearing cells were comparable in the CBA/Tufts normal, nude, and xid mice. In the three nude xid mice examined, sIg-positive cells were <2.5, 30, and 7% of the normal values. Thus, there was a striking deficit of sIg-positive cells.

*Circulating Lymphocytes.* The failure to find normal numbers of mature sIg-positive cells in the spleens of nude xid mice and the known lack of circulating T cells in nude mice (28) prompted an investigation of the number of circulating lymphocytes in doubly defective mice. Apparently, there are circulating lymphocytes, but in decreased numbers, even less than are found in nude mice (Table VI).

*Histology of Lymph Nodes.* The lack of functioning B cells and the deficit of cells with sIg suggested that these mice might lack all mature cells of the B cell lineage. Because B cells occupy well described areas of the peripheral lymphoid organs, namely the primary follicles (29), ordinary light microscopy could answer this question. Both normal and xid mice had many lymphocytes in the paracortical and cortical areas of their peripheral lymph nodes (Figs. 1 A and B). Nude mice had depleted paracortical thymus-dependent areas (30) but had many cells in the cortex (Fig. 1 C). In the nude xid mouse, few lymphocytes were found in either the paracortex or the cortex (Fig. 1 D). In particular, it should be noted that doubly defective mice have no discernable primary follicles (Fig. 2 D).

## Discussion

The evidence presented here leads to the firm conclusion that doubly deficient nude, xid mice have a deficit of mature B cells. This conclusion is based on the observations that (a) they have <5% of normal circulating Ig; (b) they fail to make antibody in vitro when presented with thymus-independent antigens; (c) they do not

TABLE VI  
Peripheral Blood Leukocyte Counts

	Phenotype			
	Normal (3)*	Nude (3)	xid (4)	Nude xid (4)
Total leukocyte count/mm <sup>3</sup> (SEM)	2,120 (± 431)	3,980 (± 1,363)	4,380 (± 1,689)	2,435 (± 782)
Lymphocyte count/mm <sup>3</sup> (SEM)	1,709 (± 453)	1,170 (± 304)	2,427 (± 878)	340 (± 146)

\* (n).

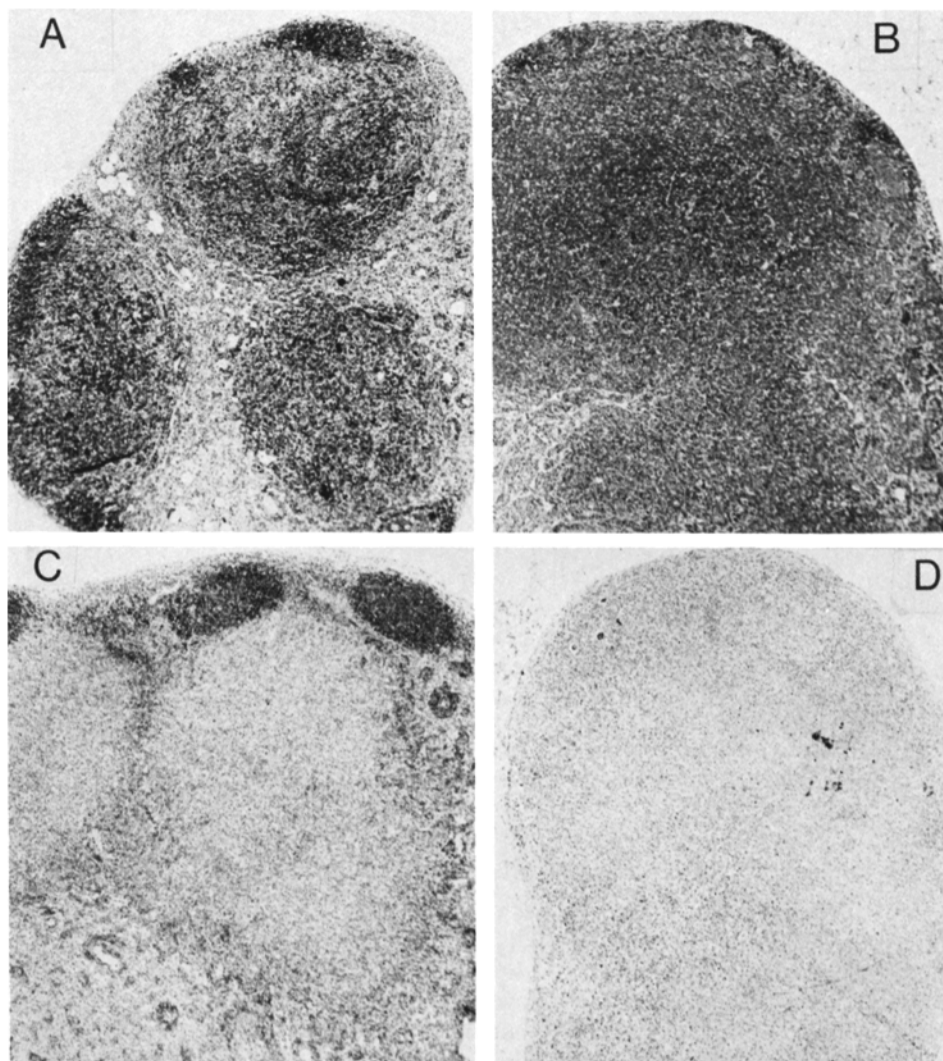


FIG. 1. Peripheral lymph nodes from (A) normal, (B) xid, (C) nude, and (D) nude xid mice. Note the depleted paracortical areas in the nude and nude xid nodes. There are few cells in cortex of the nude xid node.

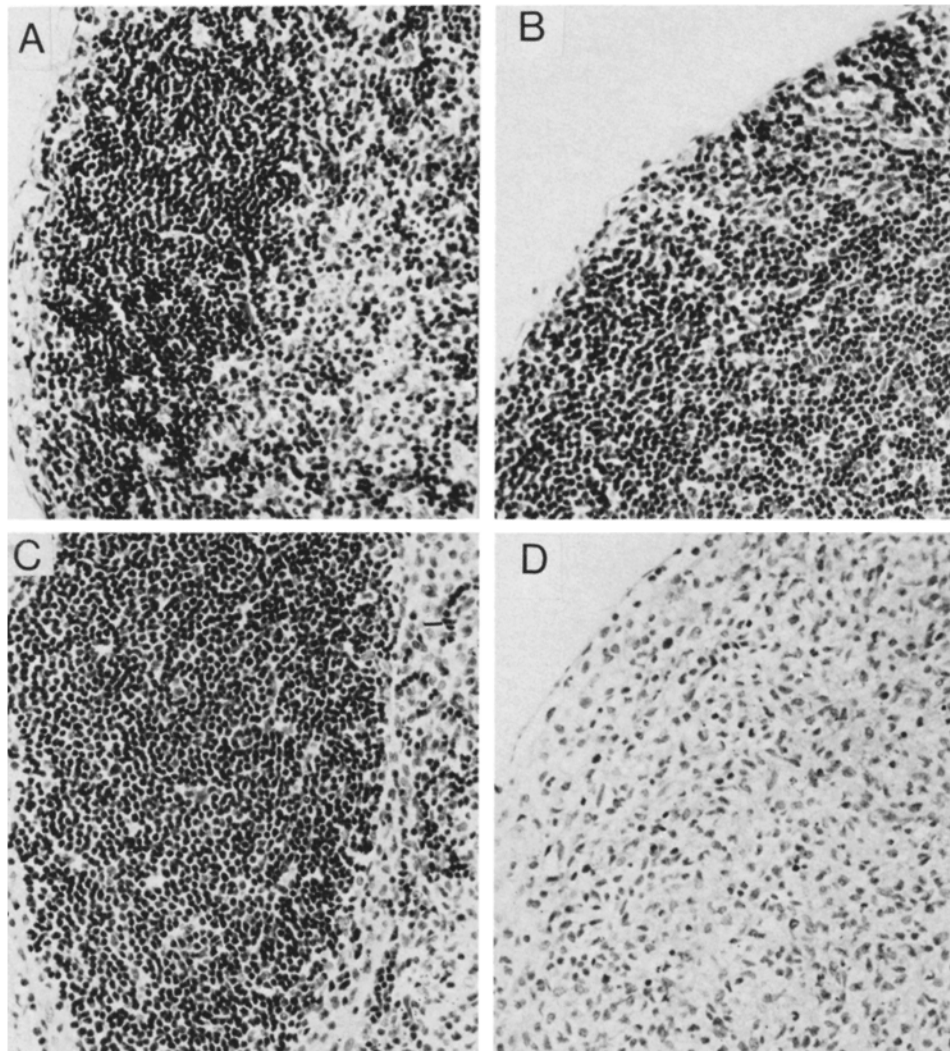


FIG. 2. The cortical region of the lymph nodes of (A) normal, (B) *xid*, (C) *nude*, and (D) *nude xid* mice. Note the absence of a primary follicle in the *nude xid* mouse.

respond to lipid A or lipoprotein of LPS, two mitogens for mature B cells (31); (*d*) they lack spleen cells with surface  $\mu$ , surface  $\kappa$ , and surface  $\lambda_1$ ; and (*e*) they have no primary B follicles.

It also seems likely, but not formally proven, that the failure of normal B cell development is a direct result of the simultaneous expression of the two recessive mutations *nude* and *xid*. The alternative explanation would be that the observed phenotype is the result of a process secondary to the expression of the double defect. For example, *nude* mice cannot mount a thymus-dependent response, and under these circumstances the further inability to respond to TI-2 antigens might lead to an infection (organism?) causing the loss of normal B cells. This seems unlikely to us because these mice survive for at least 6 mo under specific pathogen-free conditions



with contact with normal flora. Furthermore, they are known to have natural killer cells (32) and cannot be said to be devoid of all lymphocyte-based defenses. However, the placing of these mice in a strict bacteria-free environment should allow a more direct attempt to rule out this alternative hypothesis.

A more interesting hypothesis is that the combination of *nude* and *xid* produces a total failure of B cell development. Mice expressing *xid* are known to lack a population characterized by the surface markers (Lyb-3 and Lyb-5 (1, 2). It has not been established whether these cells represent a late stage in a single B cell lineage or represent one of two B cell lineages. The single lineage concept holds that there is an arrest of antigen-independent B cell maturation at a point after cytoplasmic and surface Ig are expressed but before Lyb-3 and Lyb-5 appear on the cell membrane. Thus, *xid* mice express normal relative frequencies of  $\mu$ - and  $\gamma$ -bearing B cells (33). The studies reported here indicate that B cell development in *nude xid* mice is blocked at a step earlier than the expression of surface Ig. The simplest explanation of this observation is that normal B cells do belong to two populations and that *nude* and *xid* each block the differentiation of one lineage earlier than the mature B cell stage. For this to be correct, *nude* mice must express a defect in B cell development not previously described, and the mature B cells of *xid* mice would have to belong entirely to one of two B cell lineages. Direct proof of these postulates requires the identification of cells of the two B cell lineages at the level of the pre-B or stem cell.

The single B cell lineage model could be preserved if normal B cell development depends on a differentiation step that can be accomplished by either one of two alternative pathways, one controlled by alleles at the *nude* locus and the other by the *xid* locus. The *xid* locus would also control the maturation of B cells to late [Lyb-3<sup>+</sup>] B cells.

At best, evidence of B cell defects in the *nude* has been suggestive. However, if *nude* mice do lack a normal B cell population, the interesting question arises as to the primary site of the block. *Nudes* lack mature T cells, but there is no intrinsic defect in T cell precursors, rather it is the lack of the normal inductive environment provided by the thymus that causes the block in T cell differentiation (34). The proposed block in B cell development imposed by *nude* could either be intrinsic to B cell precursors or secondary to a defect in a tissue that normally induces B cell differentiation. If a defect in inductive tissue exists, it is an entirely open question as to whether the thymus itself provides the inductive signals, for instance, by means of a hormone.

The *nude xid* mice described here resemble mice expressing the *xid* gene on a C3H/HeJ or C3H/HeN background (35) that also do not respond to TI antigens or B cell mitogens. Unlike *nude xid* mice, *xid* C3H mice can respond to thymus-dependent antigens and make both IgM and IgG antibody. Their defect is not because of the combined effects of the *xid* gene with the *Lps<sup>d</sup>* allele, but the possibility that the gene(s) causing low responses of C3H/HeN mice to LPS (36) contributes to the combined defect has not been ruled out. Perhaps the *xid* C3H mice lack Lyb-3, Lyb-5 cells but, unlike the *nude xid* mice, have a second B cell population that can be triggered by antigen only in conjunction with a T cell signal.

### Summary

Mice were bred that simultaneously expressed the mutations *nude* and *x-linked immune deficiency (xid)*. These doubly deficient animals had <10% of normal serum

immunoglobulin levels. Their spleen cells did not respond to thymus-independent antigens *in vitro* nor did they respond to lipopolysaccharide. There was a virtual absence of cells with surface  $\mu$ ,  $\kappa$ , or  $\lambda_1$ , as detected by fluorescence. Sections of lymphoid organs revealed an absence of primary B cell follicles. Taken together, these results indicate a lack of mature B cells in nude *xid* mice. The possibility is considered that mature B cells belong to two subpopulations representing two lineages, one controlled by alleles at the *xid* locus and the other by alleles at the *nude* locus.

We appreciate the help of Cheryl Stafford who prepared the manuscript and Steven Halpern, who took the photographs.

*Received for publication 13 October 1981.*

### References

1. Huber, B., R. K. Gershon, and H. Cantor. 1977. Identification of a B-cell surface structure involved in antigen-dependent triggering: absence of this structure on B cells from CBA/N mutant mice. *J. Exp. Med.* **145**:10.
2. Ahmed, A., I. Scher, S. O. Sharrow, A. H. Smith, W. E. Paul, D. H. Sachs, and K. V. Sell. 1977. B-lymphocyte heterogeneity: development and characterization of an alloantiserum which distinguishes B-lymphocyte differentiation alloantigens. *J. Exp. Med.* **145**:101.
3. Subbarao, B., D. E. Mosier, A. Ahmed, J. J. Mond, I. Scher, and W. E. Paul. 1979. Role of a nonimmunoglobulin cell surface determinant in the activation of B lymphocytes by thymus-independent antigens. *J. Exp. Med.* **149**:495.
4. Ahmed, A. and I. Scher. 1976. Studies on non-H-2 linked lymphocyte activating determinants. II. Non-expression of M1s determinants in a mouse strain with an x-linked B-lymphocyte immune defect. *J. Immunol.* **117**:1922.
5. Huber, B. T. 1979. Antigenic marker on a functional subpopulation of B cells, controlled by the I-A subregion of the H-2 complex. *Proc. Natl. Acad. Sci. U. S. A.* **76**:3460.
6. Scher, I., M. Frantz, and A. D. Steinberg. 1973. The genetics of the immune response to a synthetic double-stranded RNA in a mutant CBA mouse strain. *J. Immunol.* **110**:1396.
7. Scher, I., A. D. Steinberg, A. K. Berning, and W. E. Paul. 1975. X-linked B-lymphocyte immune defect in CBA/HN mice. I. Studies of the function and composition of spleen cells. *J. Exp. Med.* **141**:788.
8. Kincade, P. N. 1977. Defective colony formation by B lymphocytes from CBA/N and C3H/HeJ mice. *J. Exp. Med.* **145**:249.
9. Griffith, A. L., N. Catsimpoolas, M. K. Dewanjee, and H. H. Wortis. 1976. Electrophoretic separation of radio isotopically labeled mouse lymphocytes. *J. Immunol.* **117**:1949.
10. Parks, D. E., M. V. Doyle, and W. O. Weigle. 1977. Effect of lipopolysaccharide on immunogenicity and tolerogenicity of HGG in C57B1/6J nude mice: evidence for a possible B cell deficiency. *J. Immunol.* **119**:1923.
11. Perlmutter, R. M., M. Nahm, K. E. Stein, J. Slack, I. Zitron, W. E. Paul, and J. M. Davie. 1979. Immunoglobulin subclass-specific immunodeficiency in mice with an x-linked B-lymphocyte defect. *J. Exp. Med.* **149**:993.
12. Wortis, H. H. 1974. Immunological Studies of Nude Mice. In *Contemporary Topics in Immunobiology*. Vol. III. N. Warner, editor. Plenum Publishing Corporation, New York. 243.
13. Nutt, N., J. Haber, and H. H. Wortis. 1981. Influence of Igh-linked gene products on the generation of T helper cells in the response to sheep erythrocytes. *J. Exp. Med.* **153**:1225.
14. Brodeur, P. H., and H. H. Wortis. 1980. Regulation of thymus independent responses: unresponsiveness to a second challenge of TNP-Ficoll is mediated by hapten-specific antibodies. *J. Immunol.* **125**:1499.

15. Grey, H. M., J. W. Hirst, and M. Cohn. 1971. A new mouse immunoglobulin: IgG3. *J. Exp. Med.* **133**:289.
16. Geckeler, W., J. Faversham, and M. Cohn. 1978. On a regulatory gene controlling the expression of the murine  $\lambda_1$  light chain. *J. Exp. Med.* **148**:1122.
17. Boyle, W. 1968. An extension of the  $^{51}\text{Cr}$ -release assay for the estimation of mouse cytotoxins. *Transplantation.* **6**:761.
18. von Boehmer, H., and K. Shortman. 1973. The separation of different cell classes from lymphoid organs. IX. A simple and rapid method for the removal of damaged cells from lymphoid cell suspensions. *J. Immunol. Methods.* **2**:293.
19. Bayer, E., and M. Wilchek. 1974. Insolubilized biotin for the purification of avidin. *Methods Enzymol.* **34**:365.
20. Vessiere-Louveaux, F. M. Y. R., W. Hijmans, and H. R. E. Schuit. 1980. Presence of multiple isotypes on the surface of human tonsillar lymphocytes. *Eur. J. Immunol.* **10**:186.
21. Iscove, N. N., and F. Melchers. 1978. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B-lymphocytes. *J. Exp. Med.* **147**:923.
22. Mond, J. J., and I. Scher, D. E. Mosier, M. Blease, and W. E. Paul. 1978. T-independent responses in B cell-defective CBA/N mice to *Brucella abortus* and to trinitrophenyl (TNP) conjugates of *Brucella abortus*. *Eur. J. Immunol.* **8**:459.
23. McIntyre, F. C., H. W. Sievert, G. H. Barlow, R. A. Finley, and A. Y. Lee. 1967. Chemical physical and biological properties of a lipopolysaccharide from *Escherichia coli* K-235. *Biochemistry.* **6**:2363.
24. Goodman, M. G., J. M. Fidler, and W. O. Weigle. 1978. Nonspecific activation of murine lymphocytes. IV. Proliferation of a distinct, late maturing lymphocyte subpopulation induced by 2-mercaptoethanol. *J. Immunol.* **121**:1905.
25. Diamantstein, T., H. Rühl, W. Vogt, and G. Bochart. 1973. Stimulation of B-cells by dextran sulphate *in vitro*. *Immunology.* **25**:743.
26. Owen, J. T., N. E. Wright, S. Habu, M. C. Raff, and M. D. Cooper. 1977. Studies on the generation of B lymphocytes in fetal liver and bone marrow. *J. Immunol.* **118**:2067.
27. Reth, M., T. Imanishi-Kari, and K. Rajewsky. 1979. Analysis of the repertoire of anti-(4-hydroxy-3-nitro-phenyl) acetyl (NP) antibodies in C57Bl/6 mice by cell fusion.  $\text{TT}$ -characterization of idiotypes by monoclonal anti-idiotypic antibodies. *Eur. J. Immunol.* **9**:1004.
28. Wortis, H. H. 1970. Immunological responses of "nude" mice. *Clin. Exp. Immunol.* **7**:817.
29. Gutman, G. A., and I. L. Weissman. 1972. Lymphoid tissue architecture: experimental analysis of the origin and distribution of T-cells and B-cells. *Immunology.* **23**:465.
30. Parrott, D. M. V., and M. A. B. deSousa. 1966. Changes in the thymus dependent areas of lymph nodes after immunological stimulation. *Nature (Lond.)* **212**:1316.
31. Phillips, R. A., and F. Melchers. 1979. Reduced responsiveness of immature B cells to the B cell mitogen, lipoprotein. *J. Immunol.* **122**:1473.
32. Minato, N., L. Reid, H. Cantor, P. Lengyel, and B. R. Bloom. 1980. Mode of regulation of natural killer cell activity by interferon. *J. Exp. Med.* **152**:124.
33. Huber, B. T., W. E. Gathings, and M. D. Cooper. 1979. Lyb-3 A differentiation marker for a mature subset of B cells in mice. In *B Lymphocytes in the Immune Response*. M. D. Cooper, D. Mosier, I. Scher, and E. Vitteta, editors. Elsevier/North-Holland. 125.
34. Wortis, H. H., S. Nehlsen, and J. J. Owen. 1971. Abnormal development of the thymus in "nude" mice. *J. Exp. Med.* **134**:681.
35. Bona, C., J. J. Mond, and W. E. Paul. 1980. Synergistic genetic defect in B lymphocyte function. I. Defective responses to B cell stimulants and their genetic basis. *J. Exp. Med.* **151**:224.
36. Glode, L. M., and D. L. Rosenstreich. 1976. Genetic control of B cell activation by bacterial lipopolysaccharide is mediated by multiple distinct genes or alleles. *J. Immunol.* **117**:2061.