Brief Definitive Report

EVIDENCE FOR A MATURE B CELL SUBPOPULATION IN PEYER'S PATCHES OF YOUNG ADULT *xid* MICE*

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Our understanding of B lymphocyte functional heterogeneity has been greatly advanced by studies with the X-linked immunodeficient (*xid*) CBA/N mouse (1). CBA/N mice do not respond to a set of thymus-independent (TI) antigens that includes trinitrophenyl (TNP)-Ficoll and TNP-dextran (2, 3), but retain the ability to respond to other TI antigens such as TNP-*Brucella abortus* (TNP-BA) and TNP-butanol-extracted lipopolysaccharide [TNP-LPS (Bu)] (4, 5). The latter group are termed TI-1 antigens and the former, TI-2 antigens, on the basis of this response pattern. Furthermore, mitogenic responses of CBA/N B lymphocytes to LPS, polyriboinosinic-polyribocytidylic acid (poly I-C), and purified protein derivative of tuberculin (PPD) are absent or considerably below the levels of normal controls (6). In addition, the ability of these mice to respond to the thymus-dependent antigens sheep erythrocytes (SRBC) and TNP-keyhole limpet hemocyanin is impaired (7). This deficiency is most profound under limiting in vitro culture conditions where no primary response is generated against TD antigen regardless of the dose used.

The low cell numbers, increased surface immunoglobulin (sIg)M (8), and lack of differentiation markers Lyb-3, Lyb-5, and Lyb-7 (9) exhibited by B lymphocytes of CBA/N mice are also characteristic of immature cells in neonatal and young mice. Both T lymphocytes and accessory cells of the *xid* strain are normal (7, 10), and all the immune response abnormalities have been traced to the failure of the B lymphocytes to mature (9). However, several recent reports have concluded from the at least partial restoration of the immune response in aged CBA/N mice that the arrest in B lymphocyte maturation is not absolute. At 12 mo of age, CBA/N splenic lymphocytes make significant primary in vitro antibody responses to both TNP-Ficoll (6) and SRBC (7), as well as showing increased proliferative responses to LPS, poly I-C, and PPD (6). Because human X-linked immunodeficiency syndromes are similar to that observed in CBA/N mice, studies that help explain the recovery of immune responsiveness in aged *xid* mice may be useful for our understanding of the human immune deficiency.

In this report we show that at 6–8 wk of age the Peyer's patches (PP) of CBA/N

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and (CBA/N × DBA/2) F_1 male mice contain B lymphocytes capable of eliciting primary in vitro immune responses to SRBC and the TI-2 antigens TNP-Ficoll and TNP-dextran. Further, the reactive B lymphocytes are shown to be within a subpopulation that bears sIgM levels characteristic of mature cells. These results suggest that the late maturing B lymphocytes of CBA/N mice arise in the PP, and that environmental antigens and/or mitogens are responsible for the emergence of these mature cells.

Materials and Methods

Mice. CBA/N, DBA/2, (CBA/N × DBA/2)F_1, and (DBA/2 × CBA/N) F_1 mice were bred and maintained in the Core Facility for Immunocompromised Mice, Comprehensive Cancer Center, University of Alabama in Birmingham. Mice used in the majority of these studies were 6–8 wk old.

Antigens. Aminoethylcarbamylmethyl-Ficoll, aminoethylcarbamylmethyl-dextran, and their TNP derivatives were prepared by the method of Inman (11). *Escherichia coli* K235 lipopolysaccharide (LPS) was extracted with butanol-water and substituted with TNP as previously described (12). TNP-BA was the generous gift of Dr. Irwin Scher, Naval Medical Research Institute, Bethesda, MD. SRBC were obtained weekly from the Colorado Serum Company, Denver, CO.

Cell Preparations. Mouse spleens, mesenteric lymph nodes (MLN) and PP were aseptically excised. Single-cell suspensions of spleen and MLN were prepared by gently teasing the cells through a fine stainless steel screen; PP cells were prepared by a previously described enzymatic procedure (13). Cells which were to be further separated by immunofluorescent staining and fluorescence-activated cell sorting were depleted of erythrocytes by treatment with ammonium chloride lysing buffer.

Fluorescent Staining and Cell Sorting. Splenic and PP cells were washed twice in ice cold phosphate-buffered saline that contained 5% fetal calf serum by centrifugation (1,000 rpm for 10 min) and were stained (2.5×10^7 cells) with 50 μl of fluorescein isothiocyanate-conjugated rabbit IgG specific for mouse μ chains (FITC-anti-μ) (the kind gift of Dr. John F. Kearney, Dept. of Microbiology, University of Alabama in Birmingham) by incubation for 30 min on ice. After two washes with phosphate-buffered saline that contained 5% fetal calf serum, the cells were suspended to 6×10^6/ml in the same buffer for cell sorting. Sorting was performed using a FACS IV (Becton Dickinson & Co., Sunnyvale, CA) equipped with a logarithmic amplifier (Tom Nozaki, Stanford University Medical Center, Stanford, CA) and an argon-ion laser tuned to 488 nm, with 400-mW power. Because FITC-anti-μ tended to suppress anti-TNP plaque-forming cell (PFC) responses, fluorescent antibody was removed before culture by treatment with 0.25% Viokase (Grand Island Biological Company, Grand Island, NY) at 37°C for 5 min, followed by two washes with culture medium (see below).

In Vitro Cell Cultures and PFC Assays. Splenic and PP cells were resuspended in modified Mishell and Dutton medium (12) (5×10^6 cells/0.5 ml medium) and cultured in 16-mm multiwell macroculture plates (Linbro Chemical Co., Hamden, CT). Cultures were immunized with 2.5×10^6 SRBC, 0.5 μg TNP-LPS (Bu), 50 μl 1:300 dilution of a stock TNP-BA solution that contained 10^{11} organisms/ml, 0.1 μg TNP-Ficoll, or 0.1 μg TNP-dextran and incubated at 37°C with rocking in a humidified chamber that contained 7% O_2, 10% CO_2, and 83% N_2. Triplicate cultures were assayed on day 4 for anti-TNP and on day 5 for anti-SRBC PFC by the slide modification of the Jerne and Nordin hemolytic plaque method (14).

Microcultures of sorted cells were prepared by using 5×10^5 cells/0.2 ml modified Mishell and Dutton medium in flat-bottomed 96-well microculture plates (Linbro Chemical Co.). Accessory cells were provided by the addition of 5×10^5 irradiated (3,000 rad) (CBA/N × DBA/2) F_1 male splenic mononuclear cells to each well for 2 h at 37°C in 5% CO_2, followed by the removal of nonadherent cells before addition of sorted cells for culture. In some cases, irradiated thymocyte filler cells from (CBA/N × DBA/2) F_1 male mice were used to ensure uniform cell densities. These cultures were immunized with either 5 μl of a 1:300 dilution of stock TNP-BA or 0.01 μg TNP-Ficoll and were incubated at 37°C in a stationary, humidified chamber containing 7% O_2, 10% CO_2, and 83% N_2. Triplicate cultures were assayed on day 4 for anti-TNP PFC by the Cunningham-Szenberg modification of the hemolytic technique (15).

Results and Discussion

In preliminary studies, we observed that cultures of PP lymphoreticular cells from CBA/N mice, in marked contrast to those of spleen cells, gave primary in vitro anti-SRBC PFC responses. Table I shows that both PP and MLN cells from 8-wk-old CBA/N and (CBA/N × DBA/2) F_1 male, *xid* mice produced significant anti-SRBC PFC responses, whereas spleen cells from the same mice did not respond. Under identical conditions, spleen, PP, and MLN cell cultures from (DBA/2 × CBA/N)F_1, DBA/2, and (CBA/N × DBA/2)F_1 female, normal mice gave good anti-SRBC responses. These results suggest that a phenotypically normal B lymphocyte subpopulation is present in the gut-associated lymphoreticular tissue (GALT) of *xid* mice.

To further characterize B lymphocyte responsiveness of PP cells from *xid* mice, splenic and PP cell populations from (CBA/N × DBA/2) F_1 male *xid*, and female normal, mice were tested for their ability to respond to various TNP-conjugated TI antigens (Table II). The TI-1 antigens, TNP-BA and TNP-LPS (Bu), stimulated anti-TNP PFC responses in cultures of both male and female splenic cells, whereas the TI-2 antigens, TNP-Ficoll and TNP-dextran, only stimulated responses in spleen cell cultures from normal female mice. However, cultures of PP cells from *xid* mice responded to both TI-1 and TI-2 antigens, with anti-TNP PFC responses similar to those of PP cells from (DBA/2 × CBA/N)F_1, DBA/2, and (CBA/N × DBA/2)F_1 female, normal mice. This result was also obtained when using cell populations depleted of T lymphocytes by anti-Thy-1.2 and complement treatment, which indicated the absence of a T cell requirement for these responses. Enzyme dissociation did not affect in vitro responses, since Dispase-treated *xid* spleen cells failed to respond, whereas identically treated normal spleen cells gave good TI-2 responses (data not shown). These results again suggest the presence of a mature B lymphocyte subpopulation in the PP of young adult *xid* mice.

If mature B lymphocytes are present in the PP of *xid* mice, these cells should display a lower density of sIgM. To test this presupposition, PP cell cultures were treated with FITC-anti-μ and subjected to flow cytometry. As can be seen in Fig. 1, the densities of sIgM indicate the presence of two distinct subpopulations of B lymphocytes, one with a higher density of sIgM and one with a lower density corresponding to that of PP B lymphocytes of normal F_1 mice.

To evaluate the functional capabilities of the low sIgM population from F_1 PP, the sort window of the FACS was set so that the fluorescence-negative and low sIgM cells were sorted into one pool and the high sIgM cells into another (Fig. 1). Control F_1 spleen cells were sorted and cultured in an identical manner. After in vitro challenge, both the low and high sIgM spleen and PP cells from normal F_1 female mice were responsive to TNP-BA, but only the low sIgM population responded to TNP-Ficoll. Under the same culture conditions, low and high sIgM spleen and PP cells from F_1 male *xid* mice responded to TNP-BA. Consistent with the described immune deficiency in mice homozygous and hemizygous for the *xid* gene, the vast majority of spleen cells from defective male mice exhibited the high sIgM levels characteristic of immature B lymphocytes, whereas no response to TNP-Ficoll was evident in the minor low sIgM population. In contrast, the *xid* male PP cell population contained significant numbers of low sIgM cells and made substantial anti-TNP PFC responses to TNP-Ficoll.

These results clearly demonstrate that the GALT of young adult mice that express the *xid* gene possess a subpopulation of cells that display the sIgM density and

TABLE I

*In Vitro Immune Responses to SRBC by Cultures of PP, MLN, and Spleen Cells from xid and Normal Mice**

Mice	Genotype	Cell source	IgM anti-SRBC PFC per culture	
			Cell number × 10^6 *per culture* 2.5	5.0
CBA/N	*xid*	PP	328 ± 21‡	642 ± 49
		MLN	209 ± 19	389 ± 27
		Spleen	0	0
(CBA/N × DBA/2)F_1 (male)	*xid*	PP	483 ± 43	714 ± 48
		MLN	356 ± 38	593 ± 23
		Spleen	0	0
(CBA/N × DBA/2)F_1 (female)	Normal	PP	947 ± 52	1,839 ± 91
		MLN	880 ± 29	1,203 ± 45
		Spleen	715 ± 34	1,340 ± 64
DBA/2	Normal	PP	1,235 ± 41	1,876 ± 83
		MLN	711 ± 28	983 ± 37
		Spleen	852 ± 34	1,193 ± 56

* Single cell preparations (2.5 or 5.0 × 10^6/culture) of PP, MLN, and SP from *xid* and normal mice were cultured with SRBC (2.5 × 10^6) and assayed on day 5 of incubation.

‡ Values are the mean ± SEM of triplicate determination per experiment for three separate experiments. Anti-SRBC PFC responses in control, nonimmunized cultures were 4–8 for PP, 3–7 for MLN, and 0 for spleen of *xid* mice, and 5–11 for PP, 2–9 for MLN, and 8–15 for spleen of normal mice at the highest cell concentration used.

TABLE II

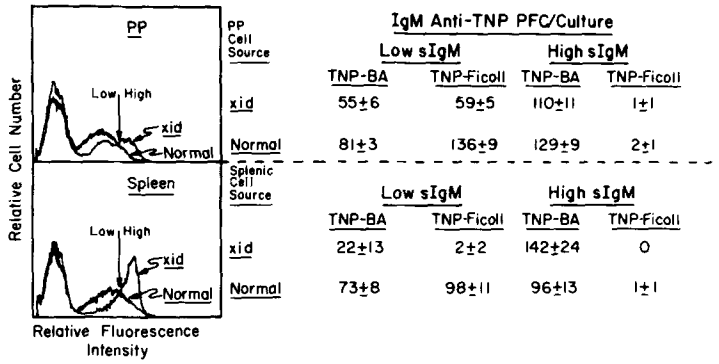
*In Vitro Immune Responses to Thymic-independent Antigens by Cultures of PP and Spleen Cells from xid and Normal Mice**

Mice [Genotype]	Cell source	Treatment	IgM anti-TNP PFC/culture			
			TNP-BA	TNP-LPS (Bu)	TNP-Ficoll	TNP-dextran
(CBA/N × DBA/2)F_1 male	PP	None	1,425 ± 89‡	912 ± 34	1,243 ± 79	1,175 ± 87
	PP	Anti-Thy 1.2 + C	1,215 ± 63	1,186 ± 72	1,045 ± 41	1,210 ± 58
[*xid*]	Spleen	None	575 ± 31	605 ± 24	0	0
	Spleen	Anti-Thy 1.2 + C	850 ± 38	537 ± 33	0	0
(CBA/N × DBA/2)F_1 female	PP	None	1,637 ± 73	1,460 ± 86	913 ± 29	1,025 ± 44
	PP	Anti-Thy 1.2 + C	1,325 ± 51	1,103 ± 63	1,112 ± 47	1,062 ± 59
[Normal]	Spleen	None	2,047 ± 87	1,970 ± 93	1,162 ± 56	1,093 ± 61
	Spleen	Anti-Thy 1.2 + C	1,300 ± 48	2,088 ± 81	1,212 ± 61	1,025 ± 53

* Single-cell preparations (5 × 10^6/culture) of PP and SP from (CBA/N × DBA/2)F_1 male and female mice were cultured with either TNP-BA (50 μl of a 1:300 dilution), TNP-LPS (Bu) (0.5 μg), TNP-Ficoll (0.1 μg), or TNP-dextran (0.1 μg), and assayed on day 4 of incubation.

‡ Values are the mean ± SEM of triplicate determinations per experiment for three separate experiments. Control, nonimmunized cultures exhibited anti-TNP PFC responses ranging from 0 to 17 per culture.

immune response characteristics of mature B lymphocytes. This subpopulation arises in the PP well before such cells appear in the spleen. Although we cannot determine from these experiments whether these cells result from an escape from regulation by the *xid* gene or represent a lineage unique to the GALT, we favor the former idea. First, in data not shown, we have observed that with increasing age (12–48 wk) the spleens of *xid* mice acquire increasing responsiveness to SRBC and TNP-Ficoll. These results suggest that the PP are the source of a natural B cell subpopulation that progressively seeds the lymphoid organs of *xid* mice as they age. Second, the GALT



PP Cell Source	IgM Anti-TNP PFC/Culture			
	Low sIgM		High sIgM	
	TNP-BA	TNP-Ficoll	TNP-BA	TNP-Ficoll
xid	55±6	59±5	110±11	1±1
Normal	81±3	136±9	129±9	2±1
Splenic Cell Source				
	Low sIgM		High sIgM	
	TNP-BA	TNP-Ficoll	TNP-BA	TNP-Ficoll
xid	22±13	2±2	142±24	0
Normal	73±8	98±11	96±13	1±1

FIG. 1. Fluorescence profiles of FITC-labeled anti-μ stained PP (upper panel) and spleen (lower panel) cells from (CBA/N × DBA/2) F_1 male (*xid*) and female (normal) mice. Cells were separated into low (negative and low) and high density sIgM pools as indicated. Low density sIgM cells (5×10^5 cells/culture) and high density sIgM cells (2.5×10^5 cells + 2.5×10^5 irradiated thymocyte cells/culture) were cultured with either TNP-BA (5 μl of a 1:300 dilution) or TNP-Ficoll (0.01 μg) and the number of IgM anti-TNP PFC per culture was determined on day 4 of incubation. Values are the mean ± SEM of triplicate determinations per experiment for three experiments.

are in intimate contact with the gut bacterial flora and the myriad of antigenic and biostimulatory substances that this environment provides. Under this influence, the maturation block may be overcome by chronic stimulation. Eventually, sufficient numbers of these mature B lymphocytes may develop and migrate from the GALT to account for the "normalized" response patterns in aged immunodeficient mice.

Support for this proposal is provided by the work of Fuhrman and Cebra (16), who used a clonal precursor assay to assess the maturation and dissemination of antigen-specific PP B cells after oral antigen administration. Their results indicate that focal priming and expansion of B cells occurs within the PP, followed by migration of the primed cells into the MLN, spleen, and distant mucosal sites. These authors proposed that this process represents a controlled reenactment of the natural priming process that commensal organisms achieve by colonization of the neonate. We feel that our results in *xid* mice and those of these authors (16) are mutually supportive of this concept.

Summary

Peyer's patch (PP) and mesenteric lymph node (MLN) cell cultures from young adult X-linked immunodeficient (*xid*) CBA/N and (CBA/N × DBA/2) F_1 male mice support primary anti-sheep erythrocyte (SRBC) plaque-forming cell (PFC) responses, which suggests that gut-associated lymphoreticular tissue (GALT) contains a normal B lymphocyte subpopulation. Further support for this was provided by the observation that PP cells from *xid* mice gave responses to both TI-1 and TI-2 antigens that were similar to the responses of PP cell cultures from normal mice. Spleen cell cultures from *xid* mice were unresponsive to SRBC and TI-2 antigens. Proof that GALT of *xid* mice contain mature B lymphocytes was provided by the demonstration of PP B cells that bear a low density of surface immunoglobulin M. When these cells were separated by flow cytometry and immunized with trinitrophenyl (TNP)-Ficoll in vitro, good anti-TNP PFC responses were observed. These results suggest that GALT of young adult *xid* mice contain mature B cells and may represent the origin for the mature B cell responses seen in aged *xid* mice.

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