ANTIBODY-DEFECTIVE, GENETICALLY SUSCEPTIBLE CBA/N MICE HAVE AN ALTERED *SALMONELLA TYPHIMURIUM*-SPECIFIC B CELL REPERTOIRE

By LISE WEISMAN DURAN and ELEANOR S. METCALF

From the Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

xid is an X-linked recessive allele carried by CBA/N mice and their hemizygous F_1 male progeny (F_1 males). This gene confers susceptibility to *Salmonella typhimurium* on these mice (1), as well as a B cell defect that results in poor or absent antibody responses to a variety of both thymus-independent (TI)[1] and thymus-dependent (TD) antigens, low preimmune serum IgM and IgG3 levels, failure to form B lymphocyte colonies in soft agar, and failure to be stimulated by antiimmunoglobulin (reviewed in references 2, 3). These abnormalities in immune function are presumed to be due to the absence of a late developing subpopulation of B cells that express the Lyb-3,5, and 7 differentiation antigens (4–6). However, T cell and macrophage/accessory cell functions are relatively normal (1, 3, 7–15). For example, with regard to immunity to *S. typhimurium*, these immune-defective mice have normal salmonella-specific, T cell–dependent delayed-type hypersensitivity (10) and the normal macrophage-mediated capacity to contain early net growth of *S. typhimurium* (1). Previous studies from this laboratory (10) suggested that susceptibility to *S. typhimurium* in mice that express the *xid* gene is a consequence of a defective anti–*S. typhimurium* antibody response. These studies showed that, when compared with normal mice, F_1 male immune-defective mice generated relatively low levels of serum anti–*S. typhimurium* antibodies after immunization with an acetone-killed and dried (AKD) preparation of *S. typhimurium*. Furthermore, passive transfer of immune serum from normal mice could protect F_1 males from a lethal challenge with live *S. typhimurium*. Taken together, these findings suggest that B cells and their antibody products are important in the development of resistance to *S. typhimu-*

This work was supported by the National Institutes of Health grant AI-22436 and AI-17755. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council Dept. of Health, Education and Welfare publication No. (NIH) 85-25, and USUHS Instruction 3203. L. W. Duran's current address is Department of Immunology, Mayo Clinic, Rochester, Minnesota 55905. Address correspondence to E. S. Metcalf, Uniformed Services University of the Health Sciences, F. Edward Hébert School of Medicine, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.

[1] *Abbreviations used in this paper:* AKD, acetone-killed and dried; KDO, 2-keto-3-deoxyoctonic acid; TD, thymus dependent; TI, thymus independent; TSA, tryptic soy agar.

rium in these mice, and that susceptibility may be a result of an altered *S. typhimurium*-specific B cell repertoire.

Recent studies from our laboratory[2] have analyzed the primary and secondary B cell repertoire specific for *S. typhimurium* in immunologically normal mice using a modified limiting dilution splenic focus assay. In the present study, we use this assay system to compare the frequency and reactivity patterns of *S. typhimurium* B cells in normal, innately resistant, CBA/Ca mice with those of the salmonella-susceptible, anti-*S. typhimurium* antibody-defective CBA/N mice. The results show: (*a*) CBA/N mice express no primary or secondary *S. typhimurium*-specific precursors; (*b*) after three immunizations, the CBA/N tertiary B cell frequency is similar to that of primary normal CBA/Ca *S. typhimurium*-specific B cells; (*c*) the percentage of CBA/N tertiary B cells specific for the LPS molecule is intermediate between that of primary and memory CBA/Ca anti-LPS antibody-producing clones; (*d*) fine specificity analysis shows a defective B cell response to the 2-keto-3-deoxyoctonic acid (KDO)/lipid A region of the LPS molecule; and (*e*) the isotypic profile in these mice is altered. Taken together, these results indicate that CBA/N mice have an abnormal distribution of *S. typhimurium*-specific B cells, and suggest that these alterations in the expressed repertoire may account for the susceptibility of these mice to *S. typhimurium*.

Materials and Methods

Animals. 6–8-wk-old CBA/Ca, AKR/J, and B10.BR mice were obtained from The Jackson Laboratory, Bar Harbor, ME. CBA/CaHN mice were obtained from the National Institutes of Health, Bethesda, MD. The two sources of CBA/Ca mice were used interchangeably. 8–10-wk-old CBA/N mice were obtained from Dominion Laboratories, Dublin, VA or NIH.

Antigens. Salmonella typhimurium strain TML was originally isolated from a patient with salmonellosis (16). Organisms were isolated on tryptic soy agar (TSA) plates (Difco Laboratories Inc., Detroit, MI) grown at 37°C. AKD bacteria of TML were prepared by the method of Landy (17) and kindly supplied by Dr. Samuel Formal, Walter Reed Army Institute of Research, Washington, D.C. LPS from TML was prepared by the procedure of Romeo et al. (18). *Streptococcus pneumoniae* type 3 was a gift of Dr. John Robbins, Bureau of Biologics, Bethesda, MD. *S. pneumoniae* was grown from a single isolated colony on blood agar plates (Difco Laboratories Inc.) for 16 h at 37°C with CO_2. *S. typhimurium* LT-2 and the LPS-deficient mutants *his*-642 (*Ra*), HN202 (*Rc*), SL1004 (*Rd1*), S11181 (*Rd2*), and TH2168 (*Re*) derived from it were the gift of Dr. J. K. Spitznagel, Emory University, Atlanta, GA. All of the bacterial cultures were grown in Penassay broth (Difco Laboratories Inc.) overnight at 37°C with shaking, from single colonies isolated on TSA plates. The actual number of organisms present in each culture was determined by serial dilution of the overnight culture in 0.15 M saline, spreading 0.1 ml on TSA plates, and overnight incubation at 37°C.

Preparation of Cell Suspensions and Adoptive Cell Transfer. For T cell depletion of donor cell populations, washed and counted spleen cell pellets were treated with 0.5 ml of anti-Thy-1.2 per spleen (hybridoma HO-13.4) (19), 0.5 ml of anti-Thy-1 framework per spleen (hybridoma T24/40.7) (20), and 1.0 ml working dilution of rabbit complement per spleen (Cederlane Laboratories Ltd., Hornby, Ontario, Canada). Treated spleen cells were incubated for 15 min at 4°C, then for 30 min at 37°C. The purity of splenic B cell suspensions obtained by T cell depletion was ascertained by immunofluorescent staining with fluorescein-conjugated anti-IgM, anti-IgD, and anti-θ and analysis on the FACS as

[2] Metcalf, E. S., M. Gaffney, and L. W. Duran. Diversity of the secondary *Salmonella typhimurium*-specific B cell repertoire. Submitted for publication.

described by Scher et al. (21). The percentage of B cells within the population was >95%.

Recipients received 10–60 × 10^6 viable untreated spleen cells or 10–25 × 10^6 T-depleted spleen cells via a lateral tail vein. Generally, pooled cells from several donors were injected into three to five recipients.

Splenic Focus Technique. The splenic focus culture system, modified for the stimulation of B cells specific for *S. typhimurium*, was previously described.[2] Briefly, primed recipients received 1,500 rad of total body irradiation from a cobalt source 4–6 h before cell transfer. AKR/J recipients were primed intravenously 14, 11, and 7 d before cell transfer with 10^3 AKD/TML organisms per mouse, and B10.BR recipients were primed intravenously one time with the same dose of AKD/TML 3 wk before. This priming regimen was necessary to optimally stimulate Th cells within the respective recipient mouse strains (data not shown). Recipients were challenged intravenously with a stimulating dose (5 × 10^7 organisms/mouse) of the inactivated preparation of TML 16 h after cell transfer. 1 h later, spleens were removed and sliced into 1-mm cube fragments on a McIlwain Tissue Chopper (Brinkman Instruments Co., Westbury, NY). The fragments were then individually placed in wells of microtiter plates (Linbro, Flow Laboratories, Inc., McLean, VA) and incubated in DME supplemented with 10% agamma horse serum and 10 mM glutamine (Grand Island Biological Co., Grand Island, NY) at 37°C in an atmosphere of 95% O_2/5% CO_2. Culture fluids were removed and replaced with 0.2 ml/well of fresh complete medium 3 d later, and the medium was changed at 3–4-d intervals thereafter. Culture fluids collected 10–23 d after stimulation were assayed for anti-TML antibody, H chain class of antibody, and fine specificity of the antibody.[2] Collected culture fluids from individual wells were stored at −20°C until time of assay.

Radioimmunoassay. The RIA of culture fluids (50 μl) for specific mouse anti-TML antibody on bacteria-coated microtiter plates was previously described (22). TML^+ culture fluids were reanalyzed in microtiter wells coated with 50 μl of purified TML-LPS diluted in phosphate buffer at a concentration of 100 ng LPS/well (23). Positive culture fluids were also reanalyzed for isotype(s) with the same RIA procedure and the following affinity-purified, class-specific rabbit anti–mouse antibodies: (*a*) anti-IgM; (*b*) anti-IgG3; (*c*) anti-IgG1; (*d*) anti-IgG2; and (*e*) anti-IgA. The preparation and specificity of these reagents have been described elsewhere (23).

Statistical Analysis. Statistical analysis was performed using Student's two-tailed *t* test for independent means.

Results

Serum Antibody Responses to AKD-TML. Immune-defective CBA/N mice have low preimmune serum IgM and IgG3 levels (24, 25). Previous studies from this laboratory (10) demonstrated that both salmonella-resistant, immunologically normal (CBA/N × DBA/2N)F_1 female and salmonella-susceptible immune-defective F_1 male mice make similar IgM anti–TML serum antibodies after immunization with AKD-TML; however, IgG titers at 3 and 4 wk after immunization were much lower in the F_1 male mice. To further characterize the anti-TML antibody response in immune-defective mice, serum samples from CBA/N mice and CBA/Ca controls were analyzed by RIA to determine the LPS specificity and the isotypes 4 wk after immunization with 5 × 10^8 AKD-TML organisms. Fig. 1*A* shows the geometric mean titers of anti-TML antibodies in CBA/Ca and CBA/N mice. These results suggest that the anti–TML IgG response in CBA/N mice is significantly ($p < 0.05$) reduced when compared with CBA/Ca mice; the data are also consistent with the results of O'Brien et al. (10) and Metcalf et al.[3]

[3] Metcalf, E. S., and A. D. O'Brien. Mice with early and late genetic defects in immune responses to *Salmonella typhimurium* can be protected by a live, avirulent vaccine. Submitted for publication.

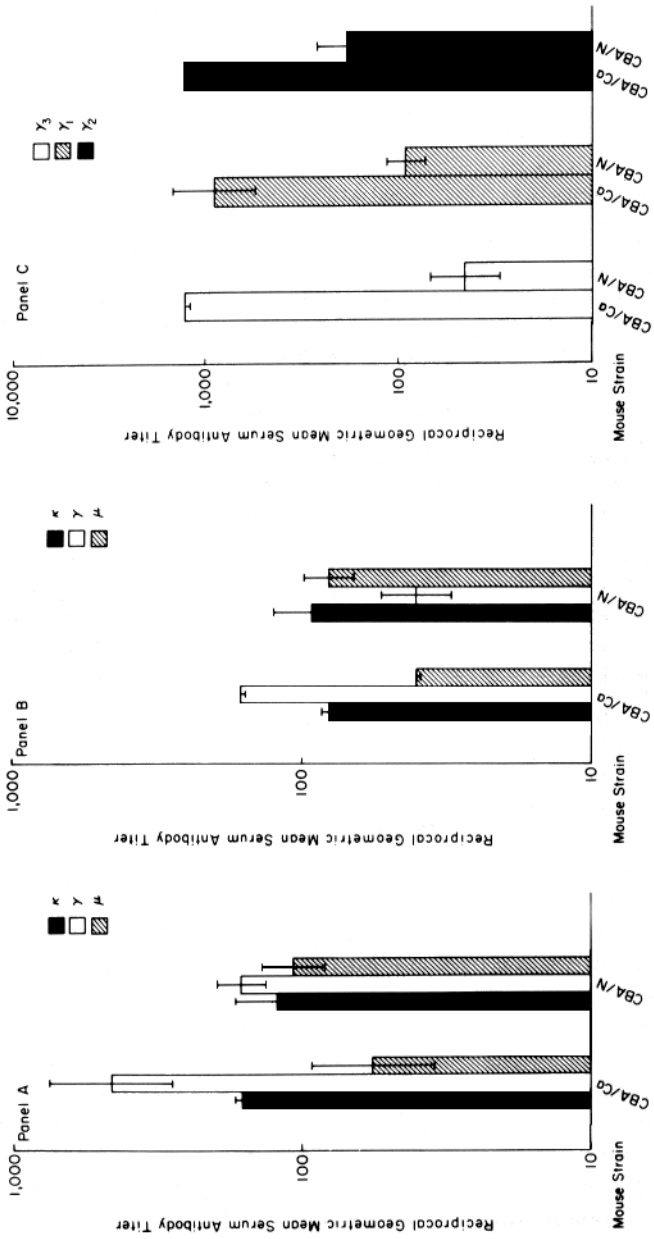

FIGURE 1. CBA/N and CBA/Ca mice were immunized intravenously with 5×10^8 AKD-TML organisms. 4 wk after immunization, serum from each mouse was assayed by RIA using whole TML organisms (*A*) or TML-LPS (*B* and *C*) as the immunoadsorbents. Anti-TML antibodies were detected with rabbit anti-mouse κ chain, IgG (all subclasses), and IgM sera (*A* and *B*) and with rabbit anti-mouse IgG3, IgG1, and IgG2 sera (*C*). Geometric mean titers ± 2 SE are given.

TABLE I

Comparison of the Frequency of Primary TML-specific B Cells in CBA/Ca and CBA/N Mice

Donor	Treatment*	Total number of cells transferred ($\times 10^{-6}$)	Number of TML-specific clones per 10^6 cells transferred‡	Number of TML-specific cells per 10^6 splenic B cells§
CBA/Ca	Anti-Thy-1 + C	30	0.27	6.68
CBA/N	—	60	0.02	0.43
CBA/N	Anti-Thy-1 + C	225	0.00	0.00

*Spleen cells were treated with T24/40.7 anti-Thy-1 plus HO-13.4 anti-Thy-1.2 plus rabbit complement as outlined in Materials and Methods.

‡ 10–25 × 10^6 anti-Thy-1 + C–treated CBA/N or CBA/Ca spleen cells and 30 × 10^6 untreated CBA/N spleens were injected into B10.BR recipients that had been previously primed intravenously with 10^3 AKD/TML organisms 3 wk before cell transfer. Stimulation of recipients and detection of clones by RIA were as described in Materials and Methods.

§ Calculated frequencies after homing efficiency and percent of B cells in the spleen were taken into account. The number of B cells in the spleens of CBA/Ca and CBA/N donor mice was 40 and 20%, respectively (7, 26).

In addition, the majority of the anti-LPS antibody activity (Fig. 1*B*) is in the IgG fraction in CBA/Ca mice and in the IgM fraction in CBA/N mice. Moreover, subclass analysis of the IgG serum component of the anti-LPS antibody response (Fig. 1*C*) shows that all subclasses are reduced ($p < 0.05$) in CBA/N mice, but the IgG3 fraction is the most reduced. These results confirm and extend the findings of O'Brien et al. (10) and are consistent with the idea that the susceptibility of mice that express the *xid* defect to *S. typhimurium* is a consequence of a delayed and diminished anti–*S. typhimurium* antibody response.

Frequency of S. typhimurium–specific B Cells. One possible explanation for the decreased antibody responses in CBA/N mice is that these mice have a reduced number of salmonella-specific precursor B cells. To address this issue, spleen cells from CBA/N and CBA/Ca mice were compared in the modified splenic focus system described in Materials and Methods. Since CBA/N mice have fewer splenic B cells (7), donor spleen cells from both strains were treated with anti-Thy-1 and C before cell transfer so that equal numbers of CBA/N and CBA/Ca B cells could be transferred to recipients. Table I shows a representative example of such an experiment. Whereas the primary *S. typhimurium* strain TML-specific precursor frequency in CBA/Ca spleens was 6.68/10^6 B cells, not a single TML-specific clone was detected in a total of 225 × 10^6 CBA/N B cells analyzed. In one experiment, we detected one clone in 60 × 10^6 CBA/N unfractionated spleen cells. However, after analysis of an additional 320 × 10^6 CBA/N spleen cells, where as many as 60 × 10^6 unfractionated cells were transferred to one recipient, we detected no other TML-specific clones (data not shown). Thus, CBA/N spleens appear to contain either very few primary Ig-secreting TML-specific B cells or none at all. To ascertain that this lack of responsiveness was not due to the preparation of *S. typhimurium* used for stimulation, CBA/N spleen cells were stimulated with a live, avirulent, temperature-sensitive mutant of TML, TS-27. Again, only one clone was found in 270 × 10^6 spleen cells analyzed (Metcalf, E. S., unpublished data).

TABLE II

Secondary and Tertiary TML-specific Precursor Frequency in CBA/Ca and CBA/N Mice

Source of Cells	Donor Priming with AKD/TML*	Total number of cells transferred ($\times 10^{-6}$)	Number of TML-specific clones per 10^6 cells transferred‡	Number TML-specific cells per 10^6 Splenic B cells§
	wk			
CBA/Ca	−6	45	1.96 ± 0.12	122.5 ± 7.3
CBA/N	−6	55	0	0
CBA/Ca	−6–2	40	2.85 ± 0.43	178.1 ± 27.0
CBA/N	−6–2	310	0.12 ± 0.01	15.4 ± 1.8

* CBA/N and CBA/Ca mice were immunized, i.v., with 10^8 AKD-TML organisms in saline either once 6 wk before, or twice, 6 and 2 wk before serving as a source of donor cells.

‡ 5×10^6 CBA/Ca or $5–20 \times 10^6$ CBA/N donor spleen cells were injected into AKD-TML-primed recipients. Priming and stimulation of recipients and detection of clones by RIA are as described in Materials and Methods. Data are presented as mean frequency ± 2 SE.

§ Calculated frequencies after homing efficiency and percent of B cells in the spleen were taken into account. The number of B cells in the spleens of CBA/Ca and CBA/N donor mice was 40 and 20%, respectively (7, 26).

In previous studies from this laboratory (27), CBA/N mice did not appear to be able to generate primary PC-specific B cells. Nevertheless, after immunization, we detected a small number of PC-specific B cell precursors in these mice. Thus, we assessed the capacity of CBA/N mice to generate secondary AKD-TML-specific B cells. Table II shows that CBA/N donor spleen cells transferred 6 wk after immunization with 10^8 AKD-TML organisms still do not give rise to detectable clones after secondary challenge. On the other hand, the secondary precursor frequency for CBA/Ca control spleen cells is 1.96 per 10^6 spleen cells, a 13-fold increase over the primary precursor frequency (27a). However, CBA/N donor spleen cells that have been immunized once 6 wk before then again 2 wk before cell transfer with 10^8 AKD/TML organisms give rise to TML-specific precursors with a frequency of 0.12 per 10^6 spleen cells after the third challenge. This frequency is 23-fold lower than the frequency obtained for tertiary TML-specific precursors in CBA/Ca controls (Table II, cf., lines *3* and *4*), but markedly similar to the primary precursor frequency in immunologically normal CBA/Ca mice[4] (also see Table V). Additional priming of CBA/N mice did not result in an increased frequency (Metcalf, E. S., data not shown). Thus, these findings are consistent with our previous studies that suggested that CBA/N B cells may need to undergo further differentiation before they can secrete antibody (27).

It is not yet clear if the inability to detect primary anti–TML precursors in CBA/N mice is due to the failure of primary CBA/N TML-specific B cells to respond in the splenic focus system or whether it is simply due to suboptimal conditions for stimulation of these B cells. This latter possibility seems unlikely because conditions for stimulation of DNP-specific precursors in immune-defective and normal mice have been shown to be identical (28). In addition, secondary PC-specific precursors and (T,G)-A--L precursors are detectable in the splenic focus system under the same conditions in which primary precursors are not detectable (3, 27).

Isotype Analysis of Anti–TML-specific mAbs. A second possible explanation for

TABLE III

Proportion of Clones from Tertiary TML-specific CBA/N B Cells Secreting Particular Isotypes

Donor Cells*	Percent of clones secreting:‡				
	IgM	IgG3	IgG1	IgG2	IgA
Primed CBA/N	8.7	0.0	28.3	73.9	13.0
Unprimed CBA/Ca	75.3	9.5	3.8	22.2	23.4
Primed CBA/Ca	71.6	23.1	35.6	57.7	44.7

* Primed CBA/N and CBA/Ca donors were immunized as described in the footnotes of Table II. TML-specific clones from primed CBA/N and CBA/Ca and unprimed CBA/Ca spleens were obtained from fragment cultures derived from AKD/TML-primed and -irradiated AKR or B10.BR recipients.

‡ Anti-TML clones were analyzed for the isotype produced by RIA against whole TML organisms using rabbit anti-mouse H chain–specific sera. Data from unprimed CBA/Ca donor cells are repeated from Duran and Metcalf (27a).

TABLE IV

Number of Isotypes Secreted by Clones from Tertiary TML-specific CBA/N B Cells

Donor cells*	Percent of individual clones secreting the following number of different isotypes:				
	1	2	3	4	5
Primed CBA/N	80.4	15.2	4.3	0	0
Unprimed CBA/Ca	72.2	21.5	5.7	0.6	0
Primed CBA/Ca	27.4	34.1	21.6	13.9	4.3

* Primed CBA/N and CBA/Ca donors were immunized as described in Table II. TML-specific clones from primed CBA/N and CBA/Ca and unprimed CBA/Ca spleens were obtained from fragment cultures derived from AKD-TML-primed AKR or B10.BR recipients. Data from unprimed CBA/Ca donor cells are repeated from Duran and Metcalf (27a).

the susceptibility of CBA/N mice to *S. typhimurium* may be an altered isotype distribution pattern. Therefore, we analyzed the isotype profile of tertiary TML-specific clones from CBA/N mice (Table III). In contrast to either the primary or memory CBA/Ca response, the majority of the CBA/N clones secrete IgG2 anti-TML antibody. Since CBA/N mice normally have low levels of serum IgG3 (24, 25) and are unresponsive to antigens that stimulate IgG3 production (29), it is not surprising that none of the CBA/N-derived clones secrete the IgG3 subclass. In addition, only a small proportion of these clones secrete either IgA or IgM anti-TML antibodies. These results indicate that, although the tertiary TML-specific precursor frequency in CBA/N mice is similar to the frequency of primary CBA/Ca B cells, the distribution of the isotypes secreted from such clones is markedly different. Further, although the CBA/N TML-specific clones can be generated after a third challenge with AKD-TML, their isotype profile is also markedly different from that of CBA/Ca memory anti-TML B cell clones.

It is also possible that an altered response to TML may be a reflection of the number of different isotypes secreted by CBA/N B cells. Therefore, in Table IV the number of different isotypes secreted by individual clones derived from CBA/N mice is compared with the number secreted from CBA/Ca primary and memory TML-specific foci. It is important to note that Poisson distribution analysis of our data indicated that the majority of fragments that secrete more

than one H chain class contain only one TML-specific B cell.[2] Much like the CBA/Ca primary anti-TML clones, most CBA/N clones secrete only one isotype with a few secreting two or three isotypes. However, differences between these two B cell subpopulations can be observed. One difference, aside from the lack of IgG3-secreting clones, is that more than half of the CBA/N IgG1 anti-TML antibodies are secreted in the absence of other isotypes, and the remainder of the clones that secrete IgG1 antibodies do so with only one additional isotype (data not shown). Conversely, CBA/Ca primary IgG1 anti-TML antibodies tend to be secreted with most of the isotypes (27a). A third difference is that the predominant isotype secreted by CBA/N clones is IgG2 (see Table III). Moreover, the majority of these clones secrete solely IgG2 anti-TML antibody. CBA/Ca primary clones, on the other hand, secrete IgG2 antibodies with at least one other isotype (27a).

As mentioned above, even after three immunizations the isotype profile of CBA/N TML-specific clones differs drastically from that of CBA/Ca memory clones. Tables III and IV indicate that CBA/Ca memory TML-specific B cells have undergone considerable differentiation after two and three immunizations. Indeed, the majority of these clones from normal mice secrete each isotype in combination with most of the other isotypes to a much higher degree than the CBA/Ca primary clones. In contrast, the majority of CBA/N clones that secrete each isotype, secrete only that isotype. The paucity of IgM anti-TML antibodies suggests that these TML-specific precursors have, indeed, differentiated into memory cells. However, the switching pathway appears to be different from that of CBA/Ca memory TML-specific B cell precursors.

Fine Specificity Analysis of Splenic TML-specific B Cells in CBA/N Mice. Previous studies have shown that bacterial carbohydrates stimulated antibody responses predominantly of the IgM and IgG3 isotypes (30), and that CBA/N mice are unresponsive to such antigens (29). Thus, it is surprising to find that CBA/N mice can elicit an LPS-specific anti-TML serum antibody response (Fig. 1). However, when the splenic TML-specific B cell repertoire is analyzed, the IgG3 subclass represents less than a quarter of the total CBA/Ca memory anti-TML antibody response (Table III) that is almost entirely LPS-specific (27a).[2] Hence, contrary to the findings of Slack et al. (29), B cell subsets other than those restricted to the IgM and IgG3 isotypes may be capable of responding to LPS, at least when presented to the host on the surface of the TML bacterium. These subsets, then, are probably responsible for the anti-LPS response in CBA/N mice. Because previous studies[2] suggest that the LPS-specific response is important in the memory response to salmonella infections in normal mice, and because CBA/N mice are susceptible to salmonella, it was of interest to determine what proportion of the total anti-TML antibody response was contributed by clones specific for LPS. Table V shows that 69.6% of the CBA/N TML-specific clones reacted with LPS in an RIA. In addition, none of these clones reacted with the negative control *S. pneumoniae* (data not shown). Therefore, although the frequency of TML-specific precursors is similar in unprimed CBA/Ca mice and primed CBA/N mice, the proportion of anti-LPS antibodies is over twofold lower in unprimed, normal CBA/Ca mice, and 22% of the anti-TML response in CBA/Ca mice is crossreactive with *S. pneumoniae*. The frequency of LPS-

TABLE V

LPS Specificity of TML$^+$ Clones from Tertiary CBA/N Spleen Cells

Donor cells*	Number of TML-specific clones per 10^6 cells transferred‡	Percent of anti-TML antibody clones reactive with TML-LPS§
Primed CBA/N	0.12	69.6
Unprimed CBA/Ca	0.15	28.6
Primed CBA/Ca	2.85	96.6

* Primed CBA/N and CBA/Ca donors were immunized as described in Table II.

‡ Frequencies for primed CBA/N and CBA/Ca cells are from Table II. Frequency for unprimed CBA/Ca spleen cells is from Duran and Metcalf.[4]

§ Anti-TML$^+$ clones in fragment cultures derived from recipients primed with AKD/TML were assayed in a solid phase RIA with either TML-LPS or *S. pneumoniae* as the immunoadsorbent.

TABLE VI

Fine Specificity of Anti-LPS Antibodies from CBA/N TML-specific Clones

	Immunoadsorbent:	Percent total LPS$^+$ clones with the following reactivity pattern:*					
	LPS	+	+	+	+	+	+
	Ra	−	+	+	+	+	+
	Rc	−	−	+	+	+	+
	Rd1	−	−	−	+	+	+
	Rd2	−	−	−	−	+	+
	Re	−	−	−	−	−	+
Source of Cells							
Primed CBA/N		67.7	12.9	0	3.2	0	16.1
Unprimed CBA/Ca		72.1	0	0	1.9	0	26.0
Primed CBA/Ca		43.7	2.0	1.5	3.0	5.5	44.2

* Anti-TML$^+$ clones reactive with LPS were reanalyzed using whole *S. typhimurium* rough mutant organisms as immunoadsorbents. Data from unprimed and primed CBA/Ca donor cells are repeated from Duran and Metcalf (27a).

specific clones in tertiary primed CBA/N mice has an intermediate value between unprimed and primed CBA/Ca mice.

The TML-specific clones from CBA/N mice shown to be reactive with LPS were analyzed further for reactivity with a series of O antigen–deficient, rough *S. typhimurium* mutants. These mutant strains synthesize an incomplete LPS molecule. For example, the *Ra* polysaccharide represents the complete basal core polysaccharide of the parental, smooth polysaccharide. However, the *Rc–Re* mutants produce progressively more incomplete core polysaccharides, with the *Re* mutant expressing only the KDO molecule and lipid A. It was anticipated that discrete patterns of reactivity could possibly elucidate the susceptibility of CBA/N mice to *S. typhimurium*. The results of such an analysis of tertiary CBA/N B cell products are compared with the responses of unprimed and primed CBA/Ca mice in Table VI. The results indicate that 67.7% of the TML-LPS-

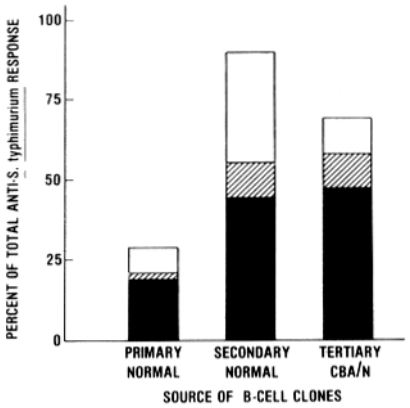

FIGURE 2. Comparison of fine specificity of LPS-specific B cell clones in normal and antibody-defective mice. TML-specific clones from primed CBA/N and from unprimed and primed CBA/Ca mice were compared by RIA using LPS and a series of *S. typhimurium* rough mutants as immunoadsorbents. The *entire bar* represents the percent of the total anti-TML B cell clones that are reactive with LPS. The *solid bar* represents the percent of the total anti-TML B cell clones that are directed to the O antigen, the *striped bar* represents the percent that are directed to core polysaccharides, and the *open area* of the bar represents the percent of clones that react with KDO/lipid A determinants.

specific precursors from primed CBA/N mice react only with the whole organism and do not react with any of the rough mutants tested. 13% of the clones recognize a site somewhere within the LPS outer core polysaccharide region (Ra^+), and 3.2% recognize a site in the inner core region ($Rd1^+$). Finally, only 16% react with all the tested rough mutants. These results are similar to the reactivity pattern observed after analysis of primary TML/LPS-specific clones from normal CBA/Ca mice. However, both responses are considerably different from the reactivity pattern of primed CBA/Ca mice. This is more clearly illustrated in Fig. 2. Whereas the majority of the primed CBA/N anti-LPS antibodies are directed against the O-antigen and only a small proportion is directed against the KDO/lipid A region, less than half of the CBA/Ca memory anti-LPS antibodies are O antigen–specific, and nearly half are KDO/lipid A–specific. Thus, the fine specificity profiles of anti-TML antibodies in CBA/N mice are distinct from those of normal mice. Alterations have also been observed in the fine specificity patterns of anti-(T,G)-A--L antibodies in CBA/N mice (3).

The results suggest that while the primed B cell subset in immune-defective CBA/N mice (presumably Lyb-5^- [5]) can produce anti-LPS antibodies, these antibodies are predominantly O antigen–specific, reminiscent of the primary TML-specific B cell repertoire. Therefore, the anti–O antigen antibodies from primed normal CBA/Ca mice presumably arise from both the Lyb-5^- and the Lyb-5^+ B cell subsets, whereas anti–lipid A antibodies primarily arise from Lyb-5^+ B cells. An analysis of additional CBA/N clones may clarify the differences in LPS reactivity between the three responses. For example, this interpretation of the results would predict that the proportion of O antigen–specific clones would increase and the proportion of lipid A–specific clones would decrease for tertiary LPS-specific CBA/N B cells. Additionally, treatment of primed CBA/Ca spleen cells with anti-Lyb-5 antibody and C should effectively diminish the anti–lipid A

component of the memory anti-TML antibody response if this hypothesis is correct.

Lyb-5$^-$ B cells also appear to respond to antigenic determinants other than LPS (Table V). Moreover, since primary TML-specific precursors from CBA/N mice do not respond in the splenic focus system and because >70% of the primary precursors from normal CBA/Ca mice do not react with LPS, it is deduced that Lyb-5$^+$ B cells also produce antibodies against other salmonellae components. Whether the reactivity patterns of these antibodies directed against other salmonellae cell surface antigens distinguishes CBA/N responses from CBA/Ca responses remains to be determined. Nonetheless, the altered fine specificity pattern of the anti-TML antibodies in these mice may influence the ability of CBA/N mice to respond to *S. typhimurium*.

Discussion

In this paper, the *S. typhimurium* strain TML-specific B cell repertoire of normal, innately resistant CBA/Ca mice was compared with that of genetically susceptible, anti-*S. typhimurium* antibody-defective CBA/N mice, which are a mutant subline of CBA/CaHN mice (2). These studies were undertaken to provide insights on the role of B cells in resistance to murine typhoid and on the genetics and immune mechanisms responsible for the differential susceptibility to this bacterial infectious disease agent. The results show that the frequency, isotype distribution patterns, and fine specificity analysis of CBA/N B cells appear to be abnormal and may account for the susceptibility of these mice to TML.

CBA/N mice do not express primary TML-specific precursors nor do they express secondary precursors after priming with AKD-TML. However, after three immunizations, the CBA/N tertiary precursor frequency is detectable. Although this frequency is similar in magnitude to that of primary CBA/Ca TML-specific B cells, the isotype profile and fine specificity of the two responses are characteristically different. Further, the tertiary CBA/N B cells appear to be memory cell–like in nature since: (*a*) very little IgM antibody is produced; (*b*) the proportion of IgG1 and IgG2 antibodies is much higher than in the primary CBA/Ca response; and (*c*) the majority of the anti-TML antibody is LPS-specific. Yet, these cells are still quantitatively and qualitatively different from CBA/Ca memory precursor cells immunized with the same regimen.

Although the majority of the memory CBA/Ca anti-TML response is LPS specific, almost half of the B cells are directed against the KDO or lipid A moiety. In contrast, only a few tertiary CBA/N clones are specific for this portion of the LPS molecule, and the majority are reactive against the O antigen region only. In addition, none of the CBA/N clones were reactive against *S. pneumoniae*. These results suggest that KDO/lipid A–specific and *S. pneumoniae*–crossreactive precursors may be restricted to the Lyb-5$^+$ B cell subset. Lyb-5$^-$ B cells, which have the characteristics of an immature B cell subset, are easily tolerizable (2, 28). Therefore, this restriction is compatible with the idea that lipid A could tolerize Lyb-5$^-$ B cells since the lipid A region is structurally similar in most gram-negative bacterial organisms (31), and because LPS is released into the surrounding environment (32). Lyb-5$^+$ B cells, on the other hand, are a mature B cell subset and are not tolerizable (2, 28).

Previous results from this laboratory offer an hypothesis that explains: (*a*) the inability to activate primary Lyb-5$^-$ B cell precursors responsive to PC in splenic focus cultures; (*b*) the ability to generate a delayed in vivo primary IgG anti-PC response but not an IgM response; and (*c*) the ability to activate in vitro secondary anti-PC B cell precursors (27). Our results suggested that PC-specific precursors in immune-defective mice must differentiate into memory cells before they can be activated to secrete antibody. We envisaged different activation requirements for Lyb-5$^-$ and Lyb-5$^+$ PC-specific cells. Consequently, we presumed that the splenic focus system may not provide all the necessary activation signals to primary Lyb-5$^-$ PC-specific B cells. The results presented here for TML-specific responses in immune-defective mice are remarkably similar to those of PC-specific responses in these mice. Thus, in accordance with this hypothesis, we can postulate that Lyb-5$^-$ TML-specific B cells, upon primary stimulation, cannot secrete antibody because they cannot receive the appropriate helper cell signal(s) that induces maturation to Ig secretion. However, these precursors can receive the helper signal(s) that activate(s) memory B cell formation. After repeated immunizations, class switching events proceed to some extent, and these Lyb-5$^-$ B cells acquire a receptor for helper cell maturation signal(s). Therefore, secretion of anti-TML antibody can begin. As opposed to the Lyb-5$^-$ B cell subset, unprimed Lyb-5$^+$ B cells, being a more mature subset (2), can secrete antibody after only a single immunization. Since Lyb-5$^+$ cells express receptors for both the proliferation signal(s) and maturation signal(s), they can secrete antibody before class-switching events have occurred. Thus, IgM anti-TML antibody is likely to be found in responses of Lyb-5$^+$ B cells but not in those of Lyb-5$^-$ B cells. This is supported by the data presented in Table III. This delay in antibody production by Lyb-5$^-$ B cells may contribute to the susceptibility of CBA/N to *S. typhimurium* infection.

Recent studies (reviewed in 33) have shown that activation of Lyb-5$^-$ and Lyb-5$^+$ B cells requires very different cellular interactions, even when responding to the same antigen and when activated by the same cloned population of Th cells. Furthermore, studies from this laboratory (34, 35), have provided evidence that suggests that T cells can supply additional proliferative signals that enhance switching events within expanding B cell clones. Moreover, the results herein suggest that although TML-specific B cell precursors in immune-defective mice may possess a receptor for the Th signal(s) that activates differentiation, isotype-switching events within these cells are mediated by a pathway(s) distinct from that of normal TML-specific B cells. It appears that after stimulation of CBA/Ca mice by AKD-TML, proliferation of an IgM$^+$ precursor cell ensues, followed by varying degrees of isotype switching within separate daughter cells of the B cell clone. Whether these switches from IgM to other isotypes occur as sequential events within one lineage of daughter cells or as distinct events within separate daughter cells is not clear. However, these data suggest that, unlike TNP/Ficoll (34, 35), AKD-TML induces distinct switching pathways since there is no apparent correlation between the frequency of clones secreting each isotype and the 5′–3′ IgCH gene order. There has been evidence that both sequential and distinct switching events can occur within clones of cells derived from single PC-specific B cells (36).

Nevertheless, whatever the mechanism, AKD-TML-stimulated normal B cells can undergo successive H chain class switches that yield expression of all five isotypes at some point in time. Upon repeated immunization, switches occur more frequently. In contrast, there appears to be only one switching pathway in immune-defective mice. Although switches to the IgG2 isotype predominate in memory CBA/Ca B cell clones, this isotype is generally secreted with other isotypes. The isotype pattern expressed by tertiary CBA/N clones indicates that a single distinct IgM → IgG2 switching pathway exists for these (putative Lyb 5^-) TML-specific B cells. Occasional switches to other isotypes may occur to a minor extent, which would account for the low levels of these isotypes observed. This implies that CBA/N B cells lack a receptor(s) for helper switch signals that mediate the expression of other isotypes. Although not examined in these studies, results of past studies (37, 38) suggest that a distinct IgE switching pathway may also exist in immune-defective mice, since these mice produced significant levels of primary IgE anti-PC serum antibody and only poor levels of IgM, IgG, and IgA anti-PC antibodies. The likelihood that independent, IgG, IgA, and IgE switch pathways can occur has been substantiated by several independent lines of evidence (34–36, 39–41). Alternatively, normal switching events within CBA/N TML-specific B cells may occur, but normal secretion of Ig by these cells may not. Woloschak[4] has provided evidence that B cells from CBA/N mice have normal levels of H chain isotype-specific mRNA accumulation and transcription. However, CBA/N resting splenic B cells were found to have a marked deficiency of mRNA encoding the secreted form of α H chain.

Collectively, these data suggest several factors that may be involved in the *xid*-conferred susceptibility of CBA/N mice to *S. typhimurium* infection. The original premise was that CBA/N mice may contain a reduced number of salmonella-specific B cell precursors. Through the use of the splenic focus system, we attempted to determine the frequency of such cells. Our results show that they do not have primary or secondary Ig-secreting B cell precursors. In addition, the frequency of tertiary immune-defective precursors is 23-fold lower than tertiary precursors in normal CBA/Ca mice. However, Clough et al. (37) have shown that after repeated immunization the frequency of PC-specific precursors in immune-defective mice was nearly equivalent to that in normal mice. The similarities between the responses to TML and PC by these mice is striking. Therefore, it was anticipated that after more immunizations, the frequency of TML-specific precursors in immune-defective mice would also begin to approach the frequency seen in normal mice. However, even after five rounds of AKD-TML challenge, the frequency within these mice increased only slightly (Metcalf, E. S., data not shown). Therefore, the delayed development of these cells appears to be a very important factor in the susceptibility of these mice to infection. It has been proposed that Lyb-5^+ PC-specific precursors are responsible for the primary in vivo response and that the Lyb-5^- subset only plays a role in the secondary in vivo anti-PC response (27). Again, the results of the TML-specific response in CBA/N mice are compatible with this hypothesis. However, it has not been shown if, in fact, primary Lyb-5^- B cells from normal CBA/Ca mice

[4] Woloschak, G. E. Immunoglobulin gene expression in *xid* mice: defective expression of secreted and membrane α-heavy chain RNA. Submitted for publication.

can respond to AKD-TML in the splenic focus system. It is possible that the Lyb-5^- B cell subset may be composed of two populations; one, which has more stringent activation requirements, is present in both CBA/Ca and CBA/N mice, and one, which has similar activation requirements as Lyb-5^+ B cells, is present only in CBA/Ca mice. Other studies (28, 42, 43) have provided evidence that supports the existence of two Lyb-5^- subsets. Instead of two distinct Lyb-5^- subsets, the *xid* gene may also exert its control on the activation of Lyb-5^- B cells. Thus, primary Lyb-5^- TML-specific B cells from normal mice would be detectable. However, this is unlikely since primary PC-specific Lyb-5^- B cells from normal mice are undetectable (27). Although Lyb-5^- B cells may be involved only in secondary responses, by themselves these B cells are apparently ineffective in providing resistance to *S. typhimurium* infection. Ostensibly, if secondary Lyb-5^+ TML-specific B cells are more easily activated, this subset would be able to keep the infecting salmonellae in check until anti-TML antibody production occurs by the Lyb-5^- B cell subset. In immune-defective mice the development of these antibodies may be too late. In addition, without anti-TML antibodies produced by Lyb-5^+ B cells, those derived from Lyb-5^- B cells may be ineffective in handling the infection.

The data presented here indicate that the anti-TML repertoire in immune-defective mice may be lacking a very important subset of TML-specific B cells. In spite of the fact that the majority of anti-TML antibodies derived from CBA/N mice are LPS specific, only a small proportion are KDO/lipid A specific. Because almost half to two-thirds of the LPS-specific anti-TML antibodies from normal, memory CBA/Ca clones are directed to KDO/lipid A (Table VI), this deficiency in immune-defective mice may be deleterious. Although anti–O antigen antibodies may be important to long-term immunity (44), these findings (summarized in Fig. 2) suggest that the development of these antibodies in immune-defective mice is not enough for protective immunity. Following from the discussion in the preceding paragraph, without the anti–lipid A antibodies that are primarily produced by Lyb-5^+ B cells, the anti–O antigen antibodies derived from Lyb-5^- B cells may not be able to control the infection. CBA/N mice also appear to be lacking another subset of TML-specific B cells, although this subset and the KDO/lipid A–specific subset may not be mutually exclusive. This missing subset of B cell precursors has the capacity to switch to and/or secrete isotypes other than IgG2. The comparison of the CBA/Ca and CBA/N repertoires demonstrates that IgG2 anti-TML antibody is predominant in the normal primary and memory responses, and that Lyb-5^- B cells are presumably responsible for the increase in IgG2 production in memory responses. However, it appears that those B cells whose clonal progeny can express other isotypes by class-switching events are also important in the memory response of normal, resistant mice.

Taken together, the studies presented in this report demonstrate that the *S. typhimurium*–specific B cell pool in salmonella-susceptible, antibody-defective CBA/N mice is, at least, skewed, and suggests that these mice may: (*a*) be missing an important subset of *S. typhimurium* B cells, those specific for the KDO/lipid A region of the LPS molecule; and (*b*) not be able to receive the appropriate signals from other cells that permit the B cells to switch to a class of antibody that is

protective. This combination of defects may be the basis for the susceptibility of CBA/N mice to *S. typhimurium*. In any case, the TML-specific B cell repertoire in CBA/N mice is different from that in CBA/Ca mice. Whether the differences outlined in this communication are the only factors involved in the susceptibility of these animals to *S. typhimurium* remains to be determined. Additional deficiencies in the CBA/N precursor pool may become apparent as B cell clones are further analyzed on a more extensive panel of purified bacterial cell surface antigens. Such studies are currently underway.

Summary

CBA/N mice, which express the X-linked immunodeficiency gene *xid*, are susceptible to *Salmonella typhimurium*. The basis for this susceptibility is currently unknown. However, previous studies (10) from this laboratory have provided evidence that susceptibility may be due to a defective anti–*S. typhimurium* antibody response. In that report we hypothesized that the defective antibody response may be a reflection of an altered *S. typhimurium*–specific B cell repertoire. In the studies described here, we have investigated this hypothesis using a modification of the in vitro splenic focus system. The frequency and characteristics of salmonella-specific B cells in normal, innately resistant, CBA/Ca mice have been compared with those of salmonella-susceptible, anti–*S. typhimurium* antibody–defective CBA/N mice. The results show that CBA/N mice express no primary or secondary *S. typhimurium*–specific B cell precursors after stimulation with an acetone-killed and dried (AKD) preparation of *S. typhimurium* strain TML. However, after three immunizations, the CBA/N tertiary frequency of 15.4 per 10^6 splenic B cells was similar to the primary precursor frequency in immunologically normal CBA/Ca mice, but 23-fold lower than the tertiary precursor frequency in CBA/Ca control mice. Moreover, CBA/N mice had an altered isotype distribution pattern after stimulation with AKD-TML. >70% of the tertiary CBA/N TML-specific B cells secreted IgG2, in contrast to either nonimmune or primed control mice. In addition, 80% of the CBA/N TML-specific B cells secreted only a single isotype, whereas the majority of B cells from primed normal mice secreted multiple isotypes. Fine specificity analysis of the TML-specific B cells indicated that the array of antigenic determinants to which CBA/N B cells could respond was restricted. Although the majority of primed CBA/Ca and primed CBA/N B cells were specific for LPS, the fine specificity pattern exhibited by CBA/N B cells was similar to that observed in unprimed normal mice, i. e., the vast majority were specific for the O antigen region of the LPS molecule. In contrast, a major portion of the LPS-specific B cells in primed CBA/Ca mice were directed against the KDO/lipid A region of the LPS molecule. Therefore, it appears that CBA/N mice lack or are unable to stimulate the B cell subset that predominates in primed, normal mice. Taken together, these studies indicate that the basis for susceptibility of CBA/N mice to *S. typhimurium* is multifactorial and suggests that the inability of some animals to respond to some infectious agents may be related to holes in their B cell repertoire.

We are very grateful to Drs. Larry Pease, Gayle Woloschak, and Alison O'Brien for their

critical reviews of this manuscript; to Ms. Shelley Wistar and Ms. Maryanne Gaffney for their technical assistance; and to Mrs. Theresa Lee and Mrs. Mary Brandt for the preparation of this manuscript.

Received for publication 3 September 1986.

References

1. O'Brien, A. D., I. Scher, G. H. Campbell, R. P. MacDermott, and S. B. Formal. 1979. Susceptibility of CBA/N mice to infection with *Salmonella typhimurium*: influence of the X-linked gene controlling B lymphocyte function. *J. Immunol.* 123:720.
2. Scher, I. 1982. The CBA/N mouse strain: an experimental model illustrating the influence of the X-chromosome on immunity. *Adv. Immunol.* 33:1.
3. Press, J. L., and C. A. Giorgetti. 1986. Clonal analysis of the primary and secondary B cell responses of neonatal, adult, and *xid* mice to (T,G)-A--L. *J. Immunol.* 137:784.
4. Huber, B., R. 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.* 135:10.
5. Ahmed, A., I. Scher, S. O. Sharrow, A. H. Smith, W. E. Paul, D. H. Sachs, and K. W. Sell. 1977. B lymphocyte heterogeneity: development and characterization of an alloantiserum which distinguishes B-lymphocyte differentiation alloantigens. *J. Exp. Med.* 145:101.
6. Subbarao, B., A. Ahmed, W. E. Paul, I. Scher, R. Lieberman, and D. E. Mosier. 1979. Lyb-7, a new B cell alloantigen controlled by genes linked to the IgG_H locus. *J. Immunol.* 122:2279.
7. Scher, I., A. Ahmed, D. M. Strong, A. D. Steinberg, 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. Janeway, C. A., Jr., and D. R. Barthold. 1975. An analysis of the defective response of CBA/N mice to T-dependent antigens. *J. Immunol.* 115:898.
9. Janeway, C. A., Jr., D. L. Bert, and D. E. Mosier. 1980. Cell cooperation during *in vivo* anti-hapten antibody responses. VI. Evidence for an allogeneic effect replacing one of two helper T cells. *Eur. J. Immunol.* 10:236.
10. O'Brien, A. D., I. Scher, and E. S. Metcalf. 1981. Genetically conferred defect in anti-salmonella antibody formation renders CBA/N mice innately susceptible to *Salmonella typhimurium* infection. *J. Immunol.* 126:1368.
11. Quintans, J., Z. S. Quan, and M. A. Arias. 1982. Mice with the *xid* defect have helper cells for T15 idiotype dominant anti-phosphorylcholine primary and secondary plaque-forming cell responses. *J. Exp. Med.* 155:1245.
12. Wicker, L. S., G. Guelde, I. Scher, and J. J. Kenny. 1983. The asymmetry in idiotype-isotype expression in the response to phosphocholine is due to divergence in the expressed repertoires of $Lyb5^+$ and $Lyb5^-$ B cells. *J. Immunol.* 131:2465.
13. Feeny, A. J., and D. E. Mosier. 1984. Helper T lymphocytes from *xid* and normal mice support anti-phosphocholine antibody responses with equivalent T15, 511, and 603 idiotypic composition. *J. Immunol.* 133:2868.
14. Boswell, H. S., S. D. Sharrow, and A. Singer. 1980. Role of accessory cells in B cell activation. I. Macrophage presentation of TNP-Ficoll: evidence for macrophage-B cell interaction. *J. Immunol.* 124:989.
15. Rosenstreich, D. L., S. N. Vogel, A. Jacques, L. M. Wahl, I. Scher, and S. E. Mergenhagen. 1978. Differential endotoxin sensitivity of lymphocytes and macrophages from mice with an X-linked defect in B cell maturation. *J. Immunol.* 121:685.
16. Gianella, R. A., S. A. Broitman, and N. Z. Zamcheck. 1971. *Salmonella enteritis*: fulminent diarrhea in and effects on the small intestine. *Am. J. Dig. Dis.* 16:1007.

17. Landy, M. 1953. Enhancement of the immunogenicity of typhoid vaccine by retention of the VI antigen. *Am. J. Hyg.* 58:148.
18. Romeo, D., A. Girard, and L. Rothfield. 1970. Reconstitution of a functional membrane enzyme system in a monomolecular film. I. Formation of a mixed monolayer of lipopolysaccharide and phospholipid. *J. Mol. Biol.* 53:475.
19. Marshak-Rothstein, A., P. Fink, J. Gridley, D. H. Raulet, M. J. Bevan, and M. L. Gefter. 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* 122:2491.
20. Dennert, G., R. Hyman, J. Lesley, and I. S. Trowbridge. 1980. Effects of cytotoxic monoclonal antibody specific for T200 glycoprotein on functional lymphoid cell populations. *Cell. Immunol.* 53:350.
21. Scher, I., S. O. Sharrow, and W. E. Paul. 1976. X-linked B-lymphocyte defect in CBA/N mice. III. Abnormal development of B-lymphocyte populations defined by their density of surface immunoglobulin. *J. Exp. Med.* 144:507.
22. Metcalf, E. S., and A. O'Brien. 1981. Characterization of murine antibody response to *Salmonella typhimurium* by a class-specific solid-phase radioimmunoassay. *Infect. Immun.* 31:33.
23. Elkins, K., and E. S. Metcalf. 1984. Monoclonal antibodies demonstrate multiple epitopes on the O-antigens of *Salmonella typhimurium* LPS. *J. Immunol.* 133:2255.
24. Amsbaugh, D. F., C. T. Hansen, B. Prescott, P. W. Stashak, D. R. Barthold, and P. J. Baker. 1972. Genetic control of the antibody response to type III pneumococcal polysaccharide in mice. I. Evidence that an X-linked gene plays a decisive role in determining responsiveness. *J. Exp. Med.* 136:931.
25. Perlmutter, R. M., M. Nahm, K. E. Stein, J. Slack, I. Zitron, E. W. Paul, and J. M. Davie. 1979. Immunoglobulin subclass-specific immunodeficiency in mice with an X-linked B-lymphocyte defect. *J. Exp. Med.* 149:993.
26. Klinman, N. R., A. R. Pickard, N. H. Sigal, P. J. Gearhart, E. S. Metcalf, and S. K. Pierce. 1976. Assessing B cell diversification by antigen receptor and precursor cell analysis. *Ann. Immunol. (Paris).* 127C:489.
27. Kenny, J. J., L. J. Yaffe, A. Ahmed, and E. S. Metcalf. 1983. Contribution of Lyb 5^+ and Lyb 5^- B cells to the primary and secondary phosphocholine-specific antibody response. *J. Immunol.* 130:2574.
27a. Duran, L. W., and E. S. Metcalf. 1987. Clonal analysis of primary *B* cells responsive to the pathogenic bacterium *Salmonella typhimurium*. *J. Exp. Med.* In press.
28. Metcalf, E. S., I. Scher, and N. R. Klinman. 1980. Susceptibility to in vitro tolerance induction of adult B cells from mice with an X-linked B-cell defect. *J. Exp. Med.* 151:486.
29. Slack, J., G. P. Der-Balian, M. Nahm, and J. M. Davie. 1980. Subclass restriction of murine antibodies. II. The IgG plaque-forming cell response to thymus-independent type 1 and type 2 antigens in normal mice and mice expressing an X-linked immunodeficiency. *J. Exp. Med.* 151:853.
30. Perlmutter, R. M., D. Hansburg, D. E. Briles, R. A. Nicolotti, and J. M. Davie. 1978. Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol.* 121:566.
31. Luderitz, O., O. Westphal, A. M. Staub, and H. Nikaido. 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharide. *In* Microbial Toxins. Vol. IV. G. Weinbaum, S. Kadis, and S. J. Ajl, editors. Academic Press, New York. 145–233.
32. Collins, F. M. 1974. Vaccines and cell-mediated immunity. *Bacteriol. Rev.* 38:371.
33. Singer, A., Y. Asano, M. Shigeta, K. S. Hathcock, A. Ahmed, C. G. Fathman, and R. J. Hodes. 1982. Distinct B cell subpopulations differ in their genetic requirements for activation by T helper cells. *Immunol. Rev.* 64:137.

34. Mongini, P. K. A., W. E. Paul, and E. S. Metcalf. 1982. T cell regulation of immunoglobulin class expression in the antibody response to trinitrophenyl-Ficoll: evidence for T cell enhancement of the immunoglobulin class switch. *J. Exp. Med.* 155:884.
35. Mongini, P. K. A., W. E. Paul, and E. S. Metcalf. 1985. IgG subclass, IgE, and IgA anti-trinitrophenyl antibody production within trinitrophenyl-Ficoll-responsive B cell clones: evidence in support of three distinct switching pathways. *J. Exp. Med.* 157:69.
36. Gearhart, P. J., J. L. Hurwitz, and J. J. Cebra. 1980. Successive switching of antibody isotypes expressed within the lines of a B cell clone. *Proc. Natl. Acad. Sci. USA.* 77:5424.
37. Clough, E. R., D. A. Levy, and J. J. Cebra. 1981. CBA/N × BALB/cJ F_1 male and female mice can be primed to express quantitatively equivalent secondary anti-phosphocholine responses. *J. Immunol.* 126:387.
38. Kishimoto, T., S. Shigemoto, T. Watenabe, and Y. Yamamura. 1979. Demonstration of phosphorylcholine-specific IgE B cells in CBA/N mice. *J. Immunol.* 123:1039.
39. Kishimoto, T., and K. Ishizaka. 1973. Regulation of antibody response in vitro. VI. Carrier-specific helper cells for IgG and IgE antibody response. *J. Immunol.* 111:720.
40. Kimoto, M., T. Kishimoto, S. Naguchi, T. Watanabe, and Y. Yamamura. 1977. Regulation of antibody response in different immunoglobulin classes. II. Induction of *in vitro* IgE antibody response in murine spleen cells and demonstration of a possible involvement of distinct T helper cells in IgE and IgG antibody responses. *J. Immunol.* 118:840.
41. Kuritani, T., and M. C. Cooper. 1982. Human B cell differentiation. I. Analysis of immunoglobulin heavy chain switching using monoclonal antiimmunoglobulin M, G, and A antibodies and pokeweed mitogen-induced plasma cell differentiation. *J. Exp. Med.* 155:839.
42. Kung, J. T., S. O. Sharrow, A. Ahmed, R. Habbersett, I. Scher, and W. E. Paul. 1982. B lymphocyte subpopulation defined by a rat monoclonal antibody, 1468. *J. Immunol.* 128:2049.
43. Ono, S., C. J. Yaffe, J. L. Ryan, and A. Singer. 1983. Functional heterogeneity of the Lyb-5 B cell subpopulation: mutant *xid* B cells and normal Lyb-5 B cells differ in their responsiveness to phenol extracted lipopolysaccharide. *J. Immunol.* 130:2014.
44. Eisenstein, T. K., and B. M. Sultzer. 1983. Immunity to *Salmonella* infection. *In* Host Defenses to Intracellular Pathogens. Advances in Experimental Medicine and Biology. Vol. 162. T. K. Eisenstein, P. Actor, and H. Friedman, editors. Plenum Publishing Corp., New York. 261–296.