

CLONAL ANALYSIS OF PRIMARY B CELLS RESPONSIVE
TO THE PATHOGENIC BACTERIUM
SALMONELLA TYPHIMURIUM

BY LISE WEISMAN DURAN AND ELEANOR S. METCALF

*From the Department of Microbiology, Uniformed Services University of the Health Sciences,
Bethesda, Maryland 20814-4799*

Salmonella typhimurium is a Gram-negative, facultative, intracellular bacterium that multiplies in the phagocytic cells of the reticuloendothelial system. Infection with this organism causes a typhoid fever-like disease in mice. The immune response to *S. typhimurium* in various inbred strains of mice has been shown to be under genetic control (1-5). Previous studies (6-11) of the mechanism(s) which regulate the development of acquired immunity to *S. typhimurium* reported that the specific protective immune response was essentially cell mediated, depending on the presence of T cells and macrophages, but not on specific antibody or B cells. However, other studies (12-20) suggested that B cells also play a significant role in the control of *Salmonella* infections.

One approach to understanding the role of B cells in immunity to *S. typhimurium* would be to analyze the repertoire of salmonella-specific B cells in normal mice and in mice that are genetically susceptible to salmonella. Until recently, no studies of the B cell repertoire specific for salmonella, nor any other bacteria, had been undertaken. Such studies would provide insights into those subsets of B cells responsive to natural, complex antigens, and, in addition, provide an experimental approach for the analysis of the humoral immune response against other infectious agents. Previous studies from this laboratory¹ have employed a modification of the B-cell limiting dilution splenic focus assay to examine the secondary anti-*S. typhimurium* B-cell repertoire in genetically resistant CBA/Ca mice. These studies defined the conditions for the generation and analysis of monoclonal antibody responses to the acetone-killed and dried *S. typhimurium* strain TML (AKD-TML).² The results indicated that the characteristics of B

This work was supported by the National Institutes of Health grants 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 DHEW publication (NIH) 85-25, and USUHS Instruction 3203. L. W. Duran's current address is Department of Immunology, Mayo Clinic, Rochester, MN 55905. Address correspondence to E. S. Metcalf, Dept. of Microbiology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.

¹ Metcalf, E. S., M. Gaffney, and L. W. Duran. The diversity of the secondary *Salmonella typhimurium*-specific B cell repertoire. Manuscript submitted for publication.

² Abbreviations used in this paper: AKD, acetone-killed and dried; DNP-Hy, DNP-hemocyanin; Fic, Ficoll; HA, influenza virus hemagglutinin; KDO, 2-keto-3-deoxyoctonic acid; TD, thymus dependent; TI, thymus independent; TSA, tryptic soy agar.

cells specific for natural antigens were different from those specific for synthetic antigens. In addition, it was shown that (a) the frequency of salmonella-specific secondary B cells is similar to that of the hemagglutinin (HA) molecule on influenza viruses; (b) the majority of clones secrete IgM; (c) other isotypes are secreted as well, but only 35% secrete IgG1; and (d) >90% of the secondary clones were specific for the major *Salmonella* surface antigenic determinant, the LPS molecule.

Since LPS is a component of many organisms found within the normal gut flora of the host (21), it might have been anticipated that *S. typhimurium* LPS-specific precursors would be tolerized by exposure to these crossreactive gut antigens. Nevertheless, our findings show that the vast majority of secondary *S. typhimurium*-responsive B cell clones are specific for LPS.¹ However, previous studies (22) have shown that secondary B cells are resistant to tolerance induction. Yet, it is possible that the *S. typhimurium*-specific B cell repertoire may be influenced by environmental antigens. To more directly address this issue, the B cell repertoire in mice that have not been deliberately primed with antigen should be analyzed. Such an analysis would provide the first information on the characteristics of a B cell repertoire specific for a complex natural antigen that overlaps with environmental antigens. Moreover, such a study will allow us to compare these results with those that occur after deliberate immunization. These studies and comparisons may provide us with insights that will be valuable in ascertaining the efficacy of various vaccines, since they will provide information on the expansion of B cells specific for particular antigenic determinants as well as on the fine specificity of the antibody products from these B cells. Hence, we have used the modification of the splenic focus system described by Metcalf et al.¹ to analyze the primary (nonimmune) splenic B cell repertoire in CBA/Ca mice that is responsive to *S. typhimurium*. These studies include a critical analysis of the antibody products of individual salmonella-specific primary B cells by isotype and fine specificity. The results show that the frequency of primary *S. typhimurium*-specific splenic B cells in CBA/Ca mice is extremely low when compared with the frequencies obtained with many chemically defined haptens, and only a small proportion of these B cells are specific for the LPS molecule. Furthermore, the predominant isotypes secreted by primary *S. typhimurium*-specific B cell clones are IgM, IgG2, and IgA, with very little IgG1 being produced. The implications of these findings are discussed.

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.

Antigens. *Salmonella typhimurium* strain TML was originally isolated from a patient with salmonellosis (23). Organisms were isolated on tryptic soy agar (TSA; Difco Laboratories Inc., Detroit, MI) plates grown at 37°C. AKD TML were prepared by the method of Landy (24) and kindly supplied by Dr. Samuel Formal, Walter Reed Army Institute of Research, Washington, D.C. The procedure of Romeo et al. (25) was used to prepare phenol-water extracted LPS from TML. *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 CO₂.

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 kind gift of Dr. J. K. Spitznagel, Emory University, Atlanta, GA. Organisms of each strain were isolated on TSA plates grown at 37°C, then single colonies were inoculated into Penassay broth (Difco Laboratories Inc.) and grown overnight at 37°C with shaking. The actual number of organisms present in each culture was determined by counting colonies derived from serial dilutions in 0.15 M saline of the overnight culture.

Splenic Focus Technique. The splenic focus culture system, modified for analyzing mAbs to *S. typhimurium*, was previously described.¹ Briefly, primed recipients received 1,500 rad of total body irradiation from a cobalt source 4–6 h before cell transfer. The optimum priming regimen for recipients had been determined in preliminary experiments. AKR/J recipients were primed intravenously 14, 11, and 7 d before cell transfer with 10³ AKD-TML organisms per mouse, and B10.BR recipients were primed one time intravenously with the same dose of AKD-TML 3 wk before. Recipients received 30–60 × 10⁶ viable spleen cells via a lateral tail vein from unprimed donors or 5 × 10⁶ spleen cells from mice that had been immunized intravenously with 10⁸ AKD-TML organisms either once, 6 wk before, or twice, 6 wk and 2 wk before using them as primed donors. Generally, pooled cells from several donors were injected into three to five recipients. Recipients were challenged intravenously with a stimulating dose (5 × 10⁷ organisms per 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 supplemented Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, NY) at 37°C in an atmosphere of 95% O₂/5% CO₂. Culture fluids were removed and replaced with 0.2 ml/well of fresh medium and 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.¹ 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 (26). 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 (27). 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 are described elsewhere (27).

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

Results

Frequency of Primary *S. typhimurium* Strain TML-specific B Cells. The conditions for optimal stimulation of TML-specific secondary B cells with AKD-TML were determined previously.¹ Preliminary studies using the same experimental system failed to demonstrate a primary TML-specific antibody response. However, Pierce and Klinman (28) previously found that an antibody-specific immunoregulatory mechanism was established in immunized recipients that suppressed the stimulation of primary B cells. This immunoregulatory circuit appeared to be allotype linked and could be circumvented if the recipients used were syngeneic at the H-2 locus but allogeneic at the Ig H chain (IgCH) genes. Such a system was used by Cancro and his colleagues (29) to study the B cell repertoire specific for influenza virus. Therefore, it was possible that primary B cells specific for TML would be suppressed if transferred to AKD-TML-primed syngeneic recip-

TABLE I
Frequency of Primary S. typhimurium-specific B Cells in Adult CBA/Ca Mice

Exp.	Total donor cells transferred ($\times 10^{-6}$)	Recipient (AKD-TML) primed	Number TML-specific clones per 10^6 spleen cells transferred*	Number TML-specific cells per 10^6 splenic B cells [†]
1	120	AKR/J	0.21 \pm 0.06	13.0 \pm 3.7
2	330	AKR/J	0.14 \pm 0.01	8.8 \pm 0.6
3	120	AKR/J	0.10 \pm 0.02	6.3 \pm 1.1
4	120	B10.BR	0.22 \pm 0.01	13.6 \pm 0.6
5	120	B10.BR	0.16 \pm 0.04	9.9 \pm 2.4
6	160	B10.BR	0.16 \pm 0.03	9.8 \pm 1.6
7	120	B10.BR	0.12 \pm 0.05	7.5 \pm 2.9

* $30\text{--}50 \times 10^6$ CBA/Ca donor spleen cells were injected into AKD-TML primed recipients. Each experiment had two to eight primed recipients. Priming and stimulation of recipients and detection of clones by RIA were described in Materials and Methods. Data are presented as mean frequency \pm 2 SE. Mean frequency for Exp. 1-3 was 0.15 ± 0.01 and for Exp. 4-7, 0.16 ± 0.09 .

[†] Calculated frequencies after homing efficiency and percent of B cells in the spleen were taken into account (30). Mean frequency for experiments 1-3 was 9.1 ± 0.5 and for experiments 4-7, 10.1 ± 0.6 .

ients. Hence, TML-specific primary CBA/Ca B cells were analyzed in the modified splenic focus system described above using either H-2 identical/allotype different, primed AKR/J or B10.BR mice as recipients.

To measure the frequency of primary clonal precursor cells specific for TML in salmonella-resistant, immunologically normal mice, $30\text{--}50 \times 10^6$ nonimmune CBA/Ca spleen cells were injected either into lethally irradiated (1,500 rad) AKR/J recipients that had been primed intravenously 14, 11, and 7 d before with 10^3 AKD-TML organisms per mouse or into lethally irradiated B10.BR recipients that had been primed intravenously one time with the same dose of AKD-TML 3 wk before. This priming regimen is necessary to optimally stimulate Th cell activity within the respective recipient mouse strains (data not shown). All recipients were subsequently injected with 5×10^7 AKD/TML organisms per mouse 1 h before preparation of fragment cultures. The results obtained from several experiments using both AKR and B10.BR recipients are presented in Table I. The frequency data, expressed directly as the number of B cell precursors per 10^6 B cells, were calculated using the donor cell cloning efficiency and the percentage of B cells in the CBA/Ca spleen (30, 31). The results show that the frequency of TML-specific B cells is quite low compared with the frequencies observed for several haptenic determinants (see reference 32), with no apparent difference between the mean number of precursors detected in AKR-derived fragment cultures and the mean number detected in B10.BR-derived fragment cultures (9.1 per 10^6 B cells and 10.1 per 10^6 B cells, respectively).

Isotype Analysis of Anti-TML mAbs. The isotype of the antibody produced by TML-specific clones obtained in the above experiments was determined by RIA

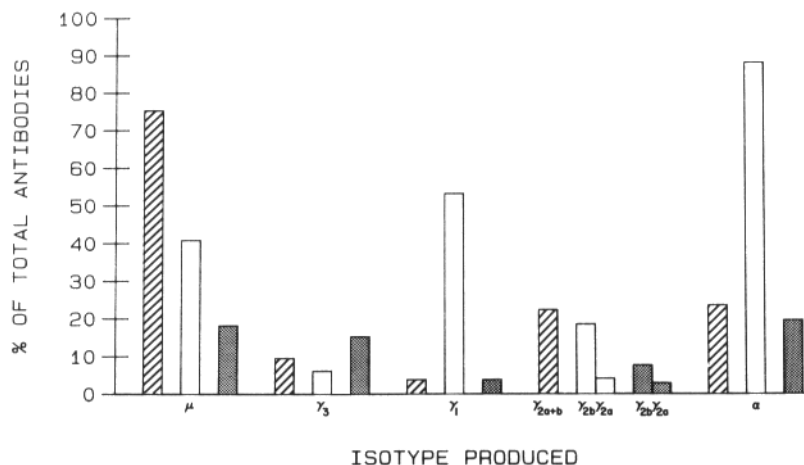


FIGURE 1. Comparison of the relative frequencies of H chain isotypes produced by primary TML-specific B cell clones with those produced by primary B cells responsive to a TD (DNP-Hy) and a TI-2 (TNP-Fic) antigen. Anti-TML antibodies were analyzed with class-specific reagents (27) as described in Materials and Methods. The relative frequency of each isotype produced in all three responses is expressed as the percent of total clones that secrete that particular isotype. 158 primary TML-specific clones were analyzed. Anti-DNP and anti-TNP antibody data are from Teale et al. (35) and Mongini et al. (37), respectively. □, DNP-Hy, ▣, TNP-Fic; ▨, TML.

using H chain class-specific rabbit anti-mouse Ig sera (27). The clones were tested using whole TML as an immunoadsorbent and usually represented a pool of day 16, 19, and 22 culture fluids. Thus, the pooled culture fluids contained the antibody produced by a single TML-specific clone during a 9–10 d period. As a consequence, mAb from such a pool may contain several isotypes due to class-switching events that may have occurred during this period of time (33–37). The percent of primary TML-specific clones that secreted a particular isotype is shown in Fig. 1. The results indicate that the predominant isotype produced is IgM followed by threefold lower levels of IgG2 and IgA. The IgG3 and IgG1 isotypes were much less frequently produced. Previous studies (38, 39) have shown that the predominant IgG subclass stimulated by thymus dependent (TD), synthetic, hapten carrier-conjugated antigens is IgG1. In contrast, type I thymus independent (TI-1) antigens were found to stimulate similar levels of IgG3 and IgG2 subclasses, and type 2 TI (TI-2) antigens were found to predominantly stimulate the IgG3 subclass (39). Fig. 1 compares the relative frequency of H chain isotypes produced by primary TML-specific clones with the frequencies produced by clones from B cells responsive to the TD antigen, DNP-Hemocyanin (DNP-Hy) and to the TI-2 antigen, TNP-Ficoll (TNP-Fic). Although significant and similar amounts of IgA are stimulated by both TML and TNP-Fic, this isotype, together with IgM and IgG3, dominates the response to the TI-2 antigen. In contrast, the expression of IgA in the TML-specific response is threefold lower than the expression of IgM and twofold higher than that of IgG3. Unlike the responses of either TI-1 or TI-2 antigens, the majority of the IgG antibody response to TML is IgG2.

However, the isotype profile of the TML-specific response is very similar to

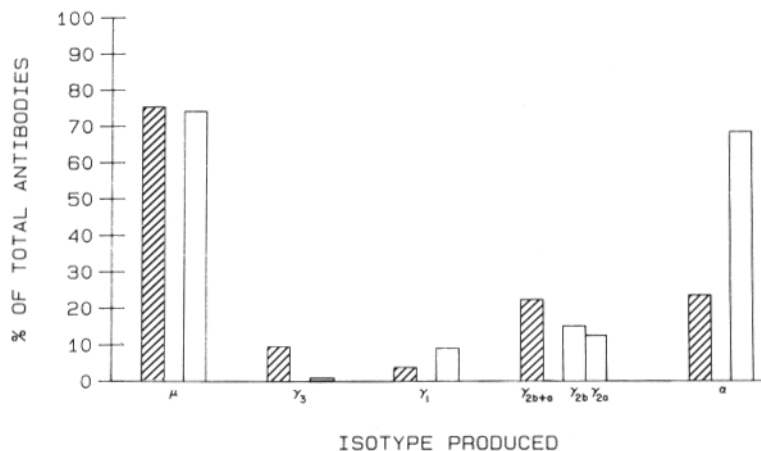


FIGURE 2. Relative frequencies of heavy chain isotypes produced by primary TML-specific and HA-specific B-cell clones. Anti-TML antibodies were analyzed with class-specific reagents (27) as described in Materials and Methods. The relative frequency of each isotype produced in both responses is expressed as the percent of total clones which secrete that particular isotype. Anti-HA antibody data is from Wylie and Klinman (40). ▨, TML; □, HA.

TABLE II
*Number of Isotypes Secreted by Clones from Primary
TML-specific Cells*

Number of clones analyzed*	Percent of individual clones secreting the following number of different isotypes:				
	1	2	3	4	5
158	72.2	21.5	5.7	0.6	0.0

* TML-specific clones from unprimed CBA/Ca donor spleen cells were obtained from fragment cultures derived from AKD-TML-primed AKR or B10.BR recipients.

the profile observed in the HA-specific response (Fig. 2). Interestingly, the isotype profile of the HA-specific response is also different from that of the DNP-Hy stimulated response (see Fig. 1, references 29, 35, 40). Taken together, these data suggest that infectious agents may either stimulate different B cell subpopulations from those stimulated by chemically defined antigens or the same B cells, but by different pathways. However, it is possible that these infectious agents may stimulate the same B cells by the same pathways. Because TML and HA are complex antigens composed of a wide array of antigenic determinants, it is possible that many of the determinants contained within the intact bacterium and virus are presented to the host in suboptimal concentrations or in an altered form compared with chemically defined antigens. Therefore, B cells specific for these determinants may not be optimally stimulated. Consequently, particular isotypes may not be optimally produced.

Table II shows the number of different isotypes secreted by individual clones from primary TML-specific B cells. The majority of these clones secrete only one isotype, but a few clones secrete two to four isotypes. It is important to note that all experiments herein were designed to ensure that each splenic fragment

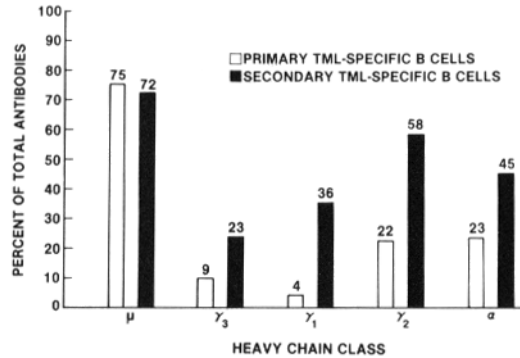


FIGURE 3. Comparison of the relative frequencies of H chain isotypes produced by primary and memory TML-specific B cell clones. Anti-TML antibodies were analyzed with class-specific reagents (27) as described in Materials and Methods. The relative frequency of each isotype produced in both responses is expressed as the percent of total clones that secrete that particular isotype. 158 primary TML-specific clones were analyzed. The memory clones analyzed represented a pool of 208 secondary and tertiary TML-specific clones that were obtained from AKD-TML primed CBA/Ca spleen cells in the splenic focus system as described in Materials and Methods. There was no difference in the isotype profile of secondary and tertiary clones.

culture contained either zero or one TML-specific B cell. Poisson distribution analysis of our data showed that <1% of the responding spleen fragments contained more than a single TML-specific B cell (data not shown). Since Table II indicates that ~30% of the fragments produce antibodies of two or more isotypes, it is likely that the majority of fragments that secrete more than one H chain class contain only one TML-specific B cell. In contrast to the TML-specific response, most clones from primary B cells stimulated by DNP-Hy or TNP-Fic secrete more than one isotype with a significant proportion of clones secreting three to six different isotypes (35–37).

Comparison of Isotype Distribution Patterns of Primary and Memory TML-specific B Cells. In previous studies¹ the isotypic profiles of memory TML-specific B cells was established. In the present study we have analyzed additional memory B cells and compared the isotype distribution pattern with that of primary TML-specific cells to determine whether particular H chain classes are selectively increased after antigen exposure. Such a finding would suggest an important role for such isotypes in the memory response to *S. typhimurium* infections. Hence, spleen cells from CBA/Ca mice previously primed with 10^8 AKD-TML were analyzed in the splenic focus system. Fig. 3 compares the isotypic distribution patterns of primary and memory anti-TML-responsive clones. In both the primary and memory responses, the predominant isotype produced is IgM, and all other isotypes increase from 2–10-fold over the primary response. Moreover, the major H chain class expressed for TML-specific B cells is IgG2, whereas IgG1 predominates in the majority of responses to synthetic antigens such as DNP-Hy (Fig. 1; references 38, 39). Nevertheless, the number of TML-specific clones that secrete IgG1 does increase 10-fold after immunization, whereas the number of IgG1 secreting DNP-specific B cells increases only twofold (35). The majority of primary TML-specific B cell clones, on the average, secrete either one or two isotypes. In contrast, a significant proportion of memory clones

TABLE III
Reactivity of Primary TML-specific Clones

Reactivity pattern number	Reactivity in RIA with:*			Number of clones [†]
	TML	LPS	<i>S. pneumoniae</i>	
1	+	+	-	30
2	+	-	-	103
3	+	+	+	12
4	+	-	+	26

* Anti-TML-positive clones in fragment cultures derived from primed AKR or B10.BR recipients were assayed by RIA with either TML-LPS or *S. pneumoniae* as the immunoadsorbents.

† A total of 171 clones were analyzed.

TABLE IV
Comparison of the LPS-reactivity of Primary and Memory TML-specific Clones

Donor cells	Total number of clones analyzed	Percent of anti-TML antibody clones reactive with TML-LPS*
Unprimed	420	28.6
TML-primed	208	96.6

* Primary and memory anti-TML-positive clones in fragment cultures derived from AKD-TML-primed AKR or B10.BR recipients were assayed by RIA using TML-LPS as the immunoadsorbent.

secreting each isotype also secrete three and four additional isotypes.¹ These findings suggest that primary TML-specific B cells do not undergo as many H chain class switches as memory B cells and appear to be consistent with other analyses of primary B cells (35).

Fine Specificity Analysis of TML-specific B Cells in Spleens of CBA/Ca Mice. *S. typhimurium* contains a multiplicity of potential antigenic determinants. The LPS molecule alone is composed of chemically diverse determinants that can elicit antibodies with a wide array of specificities. Ostensibly, antibody to those determinants that extend out from the outer membrane of the bacterium, like LPS, would make up most of the TML-specific response. Previously, it had been shown (26) that anti-LPS antibody is a major component of the anti-*S. typhimurium* response in immune sera. In addition, Metcalf et al.¹ have found that the vast majority of secondary TML-specific B cells are LPS-specific. To determine whether the majority of primary TML-specific B cells were also specific for LPS, the supernatants from primary TML-specific B cell clones were reanalyzed on LPS derived from TML. As indicated in Table III, only 24.5% of 171 TML-specific antibodies analyzed are directed against the LPS molecule. Although *S. pneumoniae* was included as a negative specificity control, 12% of the TML-specific clones are also reactive to this Gram-positive bacterium. These clones may recognize a common determinant between the two bacteria.

The results in Table IV show that only 28.6% of all the TML-specific primary

TABLE V
*Fine Specificity of Primary and Secondary LPS-reactive Clones from
 CBA/Ca Mice*

Donor cells	Number of clones analyzed	Immuno-adsorbent	Percent total LPS ⁺ clones with the following reactivity pattern*					
			LPS	Ra	Rc	Rd1	Rd2	Re
		LPS	+	+	+	+	+	+
		Ra	-	+	+	+	+	+
		Rc	-	-	+	+	+	+
		Rd1	-	-	-	+	+	+
		Rd2	-	-	-	-	+	+
		Re	-	-	-	-	-	+
Primary	104		72.1	0.0	0.0	1.9	0.0	26.0
Memory	199		43.7	2.0	1.5	3.0	5.5	44.2

* Primary and memory anti-TML-positive clones reactive with LPS were assayed by RIA using whole *S. typhimurium* rough mutant organisms as immunoadsorbents.

clones examined are specific for LPS, whereas 96.6% of the memory anti-TML antibody-producing clones are LPS specific. Moreover, previous studies¹ have shown that the frequency of memory TML-specific cells is 163.6 per 10⁶ splenic B cells. Taken together, these findings indicate that the precursor frequency expands 17-fold after antigen exposure with an apparent selective expansion of LPS-specific clones.

The LPS molecule is composed of three distinct regions, the O antigen region, the basal core region, and the lipid A region (41). The O antigen region consists of repeating units of oligosaccharides that contain specific sugars with specific linkages. Organisms that contain the entire LPS molecule are referred to as smooth strains. Rough mutant strains are blocked at different points in the biosynthesis of the LPS molecule. These mutants have been classified into chemotypes, Ra through Re, based on the sugar composition of their polysaccharides. The Ra mutant is O antigen-negative, but synthesizes the complete basal core of the parental strain, whereas the Rb to Re mutants produce core polysaccharides that have been terminated at various points in the biosynthetic pathway. The Re mutant contains the most defective polysaccharide, composed only of 2-keto-3-deoxyoctonic acid (KDO) and lipid A. TML-specific clones found to be reactive against LPS were assayed against such a series of *S. typhimurium* rough mutants in the RIA. To ensure that each of the rough mutants could act as an effective immunoadsorbent, an anti-flagellin antibody, which bound efficiently to all of the rough mutant immunoadsorbents, was included as a positive control. This analysis should indicate which subregions of the LPS molecule elicit an antibody response, as well as permit the development of a pattern of reactivity for these LPS-specific clones. The results presented in Table V show that the majority (72.1%) of the primary anti-TML-LPS-specific antibodies do not react with any of the rough mutants tested. These results suggest that the majority of the primary LPS-specific B cells are directed against the O antigen portion of the molecule since they bound only to LPS and not to the O antigen-deficient mutants. Most of the other primary LPS-specific antibodies bound to all of the

rough mutants. Only 2 of 104 clones tested appeared to be directed against sites within the core polysaccharide region of the LPS molecule. The remainder of the LPS-specific B cells are directed against sites on either the KDO region of the inner core or the lipid A region. Insufficient quantities of anti-LPS antibody from primary TML-specific clones precluded testing their reactivity against lipid A. However, recent studies from our laboratory¹ have demonstrated that the majority of secondary TML-specific B cell clones that react with the Re rough mutant listed in Table V also react with lipid A. Therefore, the results presented in Table V suggest that one-quarter of the primary TML-specific response may be specific for lipid A.

For comparison, Table V also shows the reactivity pattern of anti-LPS antibodies from memory TML-specific clones. These results indicate that less than half of the memory anti-LPS antibody response is directed against the O antigen region. Although a small proportion of the antibodies are specific for various sites within the core polysaccharide region, almost half of the antibodies are directed to the KDO/lipid A region. Therefore, taken together with the data derived from the analysis of the primary B cell clones, these studies indicate that the preferential expansion of LPS-specific B cell clones that occurs after immunization resides primarily in the B cell subset specific for the KDO/lipid A region of the LPS molecule (Table V).

Discussion

In a previous study,¹ we defined the conditions for obtaining mAb responses to AKD-TML using the splenic focus assay. This experimental system was used to characterize the responses of individual salmonella-resistant secondary precursor B cells to TML. In this paper, the primary TML-specific B cell response was analyzed and compared with the characteristics of the B cell repertoires specific for TD and TI antigens as well as memory TML-specific B cells. In conjunction with a sensitive RIA (26), these studies provide not only an estimate of the size of the expressed TML-specific B cell repertoire, but also a characterization of the isotype(s) and fine specificity of the antibody product of the clonal progeny of individual, primary TML-stimulated B cells in inherently resistant mice.

Frequency of TML-Specific B Cells. The mean frequency of TML-specific B cells in nonimmune, CBA/Ca mice of 9.6 per 10^6 splenic B cells, is extremely small when compared with the frequency of DNP-responsive cells, which is 200 per 10^6 B cells (30). In addition, this observed frequency is 15–20-fold lower than the frequency observed for several other chemically defined haptenic determinants (see reference 32). However, the frequencies of B cells responsive to other antigenic determinants on natural antigens are also several orders of magnitude lower than the frequency to the chemically defined molecules. Indeed, Cancro et al. (29) found that the frequency of influenza HA-specific B cells was, on the average, 13 per 10^6 B cells. The frequency of PC-responsive cells was determined to be 19 per 10^6 B cells (42) and that of anti-cholera toxoid antibody-producing cells was <1 per 10^6 B cells (43, 44). Cancro et al. (29) sought to explain these observations. These investigators contended that complex antigens, such as bacteria and viruses, are composed of antigenic determinants that may be very similar to self-antigens, and, as a consequence, only a small number of

determinants are recognized as foreign. Thus, the frequency of B cells specific for bacteria and viruses would be low. In contrast, simple haptenic determinants are clearly non-self structures that allow the stimulation of multiple B cell precursors. This hypothesis is attractive; however, it does not explain the low frequency of PC-specific precursors. PC, although found naturally on the cell walls of *S. pneumoniae*, is a simple haptenic determinant. Another interpretation of these studies, which would also explain responses to antigens like PC, is that clonotypes responsive to determinants on these natural antigens crossreact with antigens abundantly present in nature. In this way, most clonotypes would be tolerized during ontogeny (45–47). Metcalf and Klinman (45) provided evidence in the splenic focus system that developing B cells mature through a stage in which they are extremely susceptible to tolerance induction. Furthermore, their results suggested that the specific interaction of B cell antigen receptors with multivalent antigens (such as bacteria) is tolerogenic to neonatal (immature) B cells unless antigen is concomitantly recognized by primed T cells. Conversely, mature B cells are not tolerized by this type of interaction. Hence, most *S. typhimurium*-specific clonotypes in developing B cells would be tolerized rather than stimulated by interaction with crossreactive normal gut flora since primed T cells would not as yet be present. Only a limited subset of clonotypes, which recognize determinants distinct from environmental antigens, would be available to respond upon deliberate primary stimulation. By this argument, the frequency of primary B cells responsive to natural antigens would be expected to be low. Furthermore, secondary challenge with natural antigens would provide sufficient Th cells for stimulation of newly generated B cells emerging from the bone marrow (45, 48). Therefore, the secondary TML-specific repertoire would be expected to include those tolerizable B cell subsets that would be missing from the primary repertoire as well as those B cell subsets that would not be tolerized. Thus, the precursor frequency would be expected to increase after secondary challenge. By contrast, clonotypes specific for chemically defined determinants that are not found in nature would not be tolerized. Therefore, upon deliberate primary immunization, multiple B cell precursors for these synthetic antigens would be stimulated and the overall frequency would appear larger than the frequency for natural antigens. Hence, relative to the increase in precursor B cell frequency that occurs after challenge with natural antigens, the increase in B cell frequency after secondary challenge with synthetic antigens would seem less significant. In fact, after immunization, the frequency of TML-specific B cells increases from 1 in 10^5 splenic B cells to 1 in 6.1×10^3 splenic B cells,¹ whereas the frequency of DNP-specific B cells increases from 1 in 5×10^3 to 1 in 1.3×10^3 splenic B cells (49).

Recent studies by Thompson et al. (50, 51) demonstrated that BALB/c mice that had either been chronically or acutely immunized since the first week of life displayed repertoires most similar to that repertoire that was characteristic of the age when immunization first occurred. Therefore, antigen exposure appears to perturb the normal turnover in clonotype composition of the emerging B cell repertoire. Their results suggested this may occur by the preservation of clones that are otherwise transiently expressed, and by the prevention of the appearance of certain other HA-specific clones. Based on these studies, it would be predicted

that, early in development, many clonotypes emerging within the salmonella-specific B cell repertoire would be preserved upon contact with normal gut flora, while other clonotypes would be deleted. Furthermore, if these animals had no prior contact with crossreactive environmental antigens, the TML-specific B cell repertoire expressed by adult mice may be somewhat different. Examination of primary TML-specific precursors in germ-free mice should determine if environmental antigen exposure leads to tolerance of some salmonella-specific primary B cell precursors. Indeed, the full potential of TML-responsive clones should be expressed in these mice and, thus, a higher precursor frequency would be observed. However, primary clonotype expression in germ-free mice will also be under a state of flux, that is, distinct clonotype precursor cells would enter the B cell pool, clonally expand, and then exit from the pool at different points in time (49). Thus, even if the overall frequency increases over that in conventional mice, the expression of particular clonotypes may not change.

Isotype Analysis of TML-specific Clones. Isotype analysis of TML-specific clones showed that these responses were distinct from natural and synthetic antigen B cell responses in yet another way (35, 38). Whereas the predominant isotypes secreted by primary TML-specific B cell clones were IgM, IgG2, and IgA, classic synthetic TD antigens, e.g., DNP-Hy, stimulate predominantly IgG1 secretion (35, 38, 39). One may have expected that anti-TML antibodies would follow the same isotypic pattern as DNP-Hy (with respect to IgG1 production) since the overall response to *S. typhimurium* appears to be T cell dependent. Indeed, recent studies by this laboratory (18) reported that: (a) CBA/Ca *nu/nu* mice are susceptible to TML infection, unlike euthymic mice of the CBA/Ca background strain; and (b) CBA/Ca *nu/nu* mice are unable to produce IgM or IgG anti-*S. typhimurium* antibodies when challenged with AKD-TML. Furthermore, primary anti-TML antibody-producing clones in the splenic focus system are undetectable in fragments derived from unprimed recipients.¹ Thus, it appears that the primary response to TML is T dependent and that stimulation of primary TML-specific precursors in the splenic focus system is dependent upon the presence of antigen-primed recipient T cells. However, the isotype profile of TML-specific B cells is distinct from classical TD antigens such as DNP-Hy. It is important to note that the isotype profile is also different from the distribution typically reported for either TI-1 or TI-2 antigens (36, 37, 39). TI-1 antigens generally stimulate equivalent levels of IgG3 and IgG2 subclasses, whereas TI-2 antigens stimulate primarily the IgG3 subclass. The predominant IgG subclass stimulated by TML, on the other hand, is IgG2 only.

If IgG1 production is indicative of a classic TD response, and if the response to *S. typhimurium* is truly TD, then it may be possible that recipient Th cells are not sufficiently stimulated in the priming regimen used in these studies. Although many clones secreted anti-TML antibodies of two to four different isotypes, the majority of clones secreted predominantly IgM. This suggests that either the recipient T cells have not been optimally stimulated to help switch Ig production from the IgM isotype to other isotypes or that appropriate IgG precursors have not been expanded by T cells (52). Mongini et al. (36, 37) have provided evidence that suggests that T cells can supply additional proliferative signals which enhance switching events within expanding B cell clones. An even more comprehensive

immunization schedule may increase T cell priming that in turn may lead to an increased IgG1 anti-TML antibody response and possibly to an increased TML-specific precursor frequency. However, even with three immunizations, no significant change in T cell help was observed. Furthermore, studies by Saxen et al. (53) and Colwell et al. (54) demonstrated that IgM antibodies are protective against *S. typhimurium* challenge infections in some experimental systems.

It is also possible that the response to AKD-TML is not truly representative of the response to the viable bacterium. Acetone treatment of *S. typhimurium* may destroy or alter relevant antigenic determinants and, thus, skew the actual response. Although these concerns are particularly pertinent when assessing the B cell repertoire responsive to a complex antigen, it is interesting that the observed low frequency and distinct isotype profile of these TML-specific clones are markedly similar to those of the influenza HA-specific clones (29). The response to this complex viral antigen has been shown to be TD (29, 55, 56), yet only a small proportion of the HA-specific B cell clones produce IgG1 (29, 40). Additionally, Wylie and Klinman (40) demonstrated that the majority of HA-specific clones stimulated with an influenza-infected syngeneic cell line in splenic fragment cultures produced antibodies of the IgG2b and IgG2a subclasses. Like the response to purified influenza virus, only a small proportion of these antibodies were of the IgG1 subclass. In fact, these data may suggest the importance of IgG2 and IgA antibodies in pathogenic infections. Therefore, although much information has been gained from the study of immune responses to simple haptenic determinants, it appears that such responses cannot be generally applied to more biologically relevant antigens such as TML and influenza HA.

Fine Specificity Analysis of TML-specific Clones. Although responses to AKD-TML could be skewed, the following data suggest that this is not the case. The *S. typhimurium* antigens that elicit protective antibodies in mice have not, as yet, been identified. However, LPS is a major cell surface antigen in the murine host immune response to this infectious bacterium (21). Metcalf and O'Brien (26) have demonstrated that a major component of the anti-*S. typhimurium* antibodies in sera of mice immunized with live TML is directed against LPS. Furthermore, the O-linked polysaccharide chain of *S. typhimurium* LPS is a critical virulence factor. Hence, it is no surprise that the LPS-specific precursors are preferentially expanded upon secondary stimulation with AKD-TML. Immunization with AKD-TML reflects the apparent importance that anti-LPS antibody plays in the immune response to *S. typhimurium* infections. On the other hand, it is possible that the AKD preparation may preferentially elicit an anti-LPS response. If so, then one would expect that the primary response would also reflect a preferential LPS response. Contrary to this prediction, <30% of the anti-TML antibodies produced by primary CBA/Ca clones are LPS-specific. Therefore, AKD-TML does appear to stimulate a true representation of the TML-specific B cell repertoire or, at least, an approximation of the repertoire in terms of what is known about the role of LPS in the anti-*S. typhimurium* antibody response and in virulence (17, 26, 57-62). Furthermore, the apparent selective expansion of clones directed toward one of the major salmonella cell surface determinants (LPS) is reminiscent of the antigen-dependent expansion of influenza-specific memory B cell clones where an increase from 30% to >80% of the anti-influenza

antibodies were found to be reactive to HA, a major influenza virus determinant (29, 63). In contrast, only a two- to fourfold increase in precursor frequency occurs after immunization with simple haptenic determinants such as DNP (64). Thus, again, AKD-TML has provided patterns of stimulation similar to those of another infectious agent.

The majority of the anti-LPS antibodies derived from primary CBA/Ca TML-specific clones is directed against the O antigens. Since the O antigens are considered to be critical virulence factors (57, 58), these findings suggest that anti-O antigen antibodies may play an important role in the resistance to *S. typhimurium* infections. Interestingly, even though O antigen-specific precursors are significantly expanded after antigen exposure (from 2 per 10^6 B cells to 50–75 per 10^6 B cells), these B cells make up less than half of the total CBA/Ca memory LPS-specific B cell response. KDO/lipid A-specific precursors increase ~100-fold (from 0.7 per 10^6 B cells to 50–75 per 10^6 B cells) and make up most of the remaining one-half to two-thirds of the memory LPS-specific responses (Table V).¹ Very little of the primary anti-LPS antibody response is directed against core polysaccharide determinants and, in turn, these clones do not compose a significant proportion of the memory response. It is possible that because the core region of the LPS molecule displays considerably less chemical and antigenic diversity between strains and species of Gram-negative bacteria in comparison to O antigenic determinants (65), crossreacting antigenic exposure may have tolerized the vast majority of clones reactive to these determinants. A second possible explanation for these findings is that core determinants may be concealed by the O antigen polysaccharide and, as a consequence, stimulation of B cell precursors specific for these determinants is prevented. Furthermore, the O antigen, core polypolysaccharide, or other cell wall components may conceal lipid A antigenic sites as well. Hence, LPS released from the cell wall (11) probably serves as the immunogen for lipid A-specific B cell clones. The results presented here suggest that antibodies to the core polysaccharide region of the LPS molecule are not as important in the memory response to *S. typhimurium* infections as are anti-O antigen and anti-lipid A antibodies. Interestingly, recent studies from this laboratory suggest that salmonella-susceptible CBA/N mice which have been shown to be antibody defective (18), may have an altered repertoire to these determinants (66).

The studies presented herein contribute to the understanding of humoral immune mechanisms that may ultimately be involved in protection against an infectious disease agent. The AKD preparation as well as the conditions of the system used to analyze TML-specific precursors in CBA/Ca mice were able to stimulate a wide array of TML-specific B cells. Although the precursors that were stimulated may not represent the entire TML-specific B cell pool, both primary CBA/Ca and memory CBA/Ca B cells were studied and compared under the same conditions. Under these conditions, the isotype profile and fine specificity analysis of the TML-specific clones reflect differences among these two B cell subpopulations. In addition, the responsiveness to AKD-TML by normal mice reported in this paper is compatible to the responses to two other natural antigens, HA and PC. The precursor frequency specific for each one of these three antigens is very low in normal mice. After immunization, there is

approximately a 20-fold increase in both the TML-specific and HA-specific B cell repertoires (29, 63). Furthermore, neither TML nor HA stimulate a prominent IgG1 response, but IgG2 and IgA appear to be important in responses to natural antigens.

The similarities in B cell responsiveness to different natural antigens support the validity of the findings presented in this paper. It is hoped that these studies will provide a fundamental framework with which to further dissect and characterize the biologically relevant murine B cell repertoire to *S. typhimurium* and to other infectious disease agents. The use of a viable organism and its components in future studies should provide additional insights. Moreover, it is possible that as the response is separated into its constituent parts, a portion of the response may in fact be typical of classical responses to synthetic antigens. These studies are currently underway.

Summary

In the present study, a modification of the splenic focus system is used to analyze the *S. typhimurium* strain TML (TML)-specific B cell repertoire. The results show that the frequency of primary TML-specific splenic B cells in CBA/Ca mice is approximately 1 per 10^5 B cells and <30% of these B cells are specific for LPS. In contrast, the frequency of memory TML-specific cells is ~1 per $5-8 \times 10^3$ splenic B cells and >95% of these B cells are specific for LPS. These results suggest that the frequency of primary TML-specific B cells is extremely low and that it expands 15–20-fold after antigen exposure. It is interesting that <30% of the primary B cells are specific for the LPS molecule since it is considered to be the major antigenic determinant on *Salmonella* organisms. Furthermore, the majority of the LPS-specific anti-TML antibody-producing clones are directed against the LPS O antigen region. Conversely, more than half to two-thirds of the memory LPS-specific anti-TML B cell clones are directed against the KDO or lipid A region of the LPS molecule. These results indicate that the preferential expansion of LPS-specific B cell clones observed after immunization resides primarily in the B cell subsets responsive to the KDO/lipid A moieties on the LPS molecule. Finally, unlike B cell responses to chemically defined antigens, TML stimulates very little IgG1 antibody. IgG2 and IgA isotypes appear to play a predominant role in anti-TML antibody responses, although all H chain classes are produced to some extent. Collectively, these findings are consistent with the responses reported for two other natural antigens, HA and PC. Hence, the pattern of stimulation by infectious agents, such as *S. typhimurium*, appears to be distinct from that of synthetic antigens. Thus, the studies presented herein have begun to provide insights into those subsets of B cells responsive to *S. typhimurium* and other infectious disease organisms.

We thank Drs. Larry Pease, Gayle Woloschak, and Alison O'Brien for their critical reviews of this manuscript. We also thank Ms. Shelley Wistar and Ms. Maryanne Gaffney for their technical assistance. In addition, we gratefully acknowledge Mrs. Theresa Lee and Mrs. Mary Brandt for their help in the preparation of this manuscript.

Received for publication 5 August 1986 and in revised form 14 October 1986.

References

1. Robson, H. G., and S. I. Vas. 1972. Resistance of inbred mice to *Salmonella typhimurium*. *J. Infect. Dis.* 126:378.
2. Plant, J., and A. A. Glynn. 1976. Genetics of resistance to infection with *Salmonella typhimurium* in mice. *J. Infect. Dis.* 133:72.
3. Hormaeche, C. E. 1979. Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. *Immunology.* 37:311.
4. O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. MacDermott, and S. B. Formal. 1980. Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. *J. Immunol.* 124:20.
5. 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.
6. Mackaness, G. B., R. V. Blanden, and F. M. Collins. 1966. Host-parasite relations in mouse typhoid. *J. Exp. Med.* 124:573.
7. Blanden, R. V., G. B. Mackaness, and F. M. Collins. 1966. Mechanisms of acquired resistance in mouse typhoid. *J. Exp. Med.* 124:585.
8. Collins, F. M., G. B. Mackaness, and R. V. Blanden. 1966. Infection-immunity in experimental Salmonellosis. *J. Exp. Med.* 124:601.
9. Collins, F. M., and G. B. Mackaness. 1968. Delayed hypersensitivity and arthus reactivity in relation to host resistance in *Salmonella*-infected mice. *J. Immunol.* 101:830.
10. Collins, F. M. 1969. Effect of specific immune mouse serum on the growth of *Salmonella enteritidis* in nonvaccinated mice challenged by various routes. *J. Bacteriol.* 97:667.
11. Collins, F. M. 1974. Vaccines and cell-mediated immunity. *Bacteriol. Rev.* 38:371.
12. Jenkin, C. R., D. Rowley, and I. Auzins. 1964. The basis for immunity to mouse typhoid. I. The carrier state. *Aust. J. Exp. Biol. Med. Sci.* 42:215.
13. Jenkin, C. R., and D. Rowley. 1965. Partial purification of the "protective" antigen of *Salmonella typhimurium* and its distribution amongst various strains of bacteria. *Aust. J. Biol. Med. Sci.* 43:65.
14. Angerman, C. R., and T. K. Eisenstein. 1978. Comparative efficacy and toxicity of a ribosomal vaccine, acetone-killed cells, lipopolysaccharide, and a live cell vaccine prepared from *Salmonella typhimurium*. *Infect. Immun.* 19:575.
15. Angerman, C. R., and T. K. Eisenstein. 1980. Correlation of the duration and magnitude of protection against *Salmonella* infection afforded by various vaccines with antibody titers. *Infect. Immun.* 27:435.
16. Kuusi, N., M. Nurminen, H. Saxen, and P. H. Makela. 1981. Immunization with major outer membrane protein (porin) preparations in experimental murine Salmonellosis: Effect of lipopolysaccharide. *Infect. Immun.* 34:328.
17. Svenson, S. B., M. Nurminen, and A. A. Lindberg. 1979. Artificial *Salmonella* vaccines: O-antigenic oligosaccharide protein conjugates induce protection against infection with *Salmonella typhimurium*. *Infect. Immun.* 25:863.
18. 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.
19. Morris, J. A., C. Wray, and W. J. Sojka. 1976. The effect of T and B lymphocyte depletion on the protection of mice vaccinated with a Gal E mutant of *Salmonella typhimurium*. *Br. J. Exp. Pathol.* 57:354.
20. Hochadel, J. F., and K. F. Keller. 1977. Protective effects of passively transferred

- immune T- or B-lymphocytes in mice infected with *Salmonella typhimurium*. *J. Infect. Dis.* 135:813.
21. Sonnenwirth, A. C. 1973. The enteric bacilli and similar gram-negative bacteria. In *Microbiology*. B. D. Davis, R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, editors. Harper & Row, Publishers Inc., New York. 652-655.
 22. Pierce, S. K., E. S. Metcalf, and N. R. Klinman. 1979. Factors affecting the triggering of the B-cell repertoire. In *Cells of Immunoglobulin Synthesis*. B. Pernis, and H. J. Vogel, editors. Academic Press, New York. 253-260.
 23. Gianella, R. A., S. A. Broitman, and N. Z. Zamcheck. 1971. *Salmonella enteritis*: fulminant diarrhea in and effects on the small intestine. *Am. J. Dig. Dis.* 16:1007.
 24. Landy, M. 1953. Enhancement of the immunogenicity of typhoid vaccine by retention of the VI antigen. *Am. J. Hyg.* 58:148.
 25. 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.
 26. 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.
 27. Elkins, K., and E. S. Metcalf. 1984. Monoclonal antibodies demonstrate multiple epitopes on the O-antigens of *Salmonella typhimurium* LPS. *J. Immunol.* 133:2255.
 28. Pierce, S. K., and N. R. Klinman. 1977. Antibody-specific immunoregulation. *J. Exp. Med.* 146:509.
 29. Cancro, M. P., W. Gerhard, and N. R. Klinman. 1978. The diversity of the influenza-specific primary B-cell repertoire in BALB/c mice. *J. Exp. Med.* 147:776.
 30. 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)*. 127:489.
 31. 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.
 32. Sigal, N. H., and N. R. Klinman. 1978. The B-cell clonotype repertoire. *Adv. Immunol.* 26:255.
 33. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1975. Production of antibodies of identical idiotype but diverse immunoglobulin classes by cells derived from a single stimulated B cell. *Proc. Natl. Acad. Sci. USA.* 72:1707.
 34. 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.
 35. Teale, J. M., D. Lafrenz, N. R. Klinman, and S. Strober. 1981. Immunoglobulin class commitment exhibited by B lymphocytes separated according to surface isotype. *J. Immunol.* 126:1952.
 36. 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.
 37. Mongini, P. K. A., W. E. Paul, and E. S. Metcalf. 1983. 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.
 38. Press, J. L., and N. R. Klinman. 1973. Monoclonal production of both IgM and IgG₁ anti-hapten antibody. *J. Exp. Med.* 135:300.
 39. 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.
40. Wylie, D., and N. R. Klinman. 1981. The murine B cell repertoire responsive to an influenza-infected syngeneic cell line. *J. Immunol.* 127:194.
 41. 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.
 42. Sigal, N. H., P. J. Gearhart, and N. R. Klinman. 1975. The frequency of phosphorylcholine-specific B cells in conventional and germfree BALB/c mice. *J. Immunol.* 68:1254.
 43. Fuhrman, J. A., and J. J. Cebra. 1981. Special features of the priming process for a secretory IgA response. B cell priming with cholera toxin. *J. Exp. Med.* 153:534.
 44. Cebra, J. J., J. A. Fuhrman, P. J. Gearhart, J. L. Hurwitz, and R. D. Shahin. 1982. B lymphocyte differentiation leading to a commitment to IgA expression may depend on cell division and may occur during antigen-stimulated clonal expansion. In *Recent Advances in Mucosal Immunity*. W. Strober, L. A. Hanson, and K. W. Sell, editors. Raven Press, New York. 155-171.
 45. Metcalf, E. S., and N. R. Klinman. 1976. In vitro tolerance induction of neonatal murine B cells. *J. Exp. Med.* 143:1327.
 46. Metcalf, E. S., A. F. Schrater, and N. R. Klinman. 1979. Murine models of tolerance induction in developing and mature B cells. *Immunol. Rev.* 43:143.
 47. Teale, J. M., and N. R. Klinman. 1980. Tolerance as an active process. *Nature (Lond.)* 288:385.
 48. Metcalf, E. S., and N. R. Klinman. 1977. In vitro tolerance induction of bone marrow cells: A marker for B cell maturation. *J. Immunol.* 118:2111.
 49. Klinman, N. R., and J. L. Press. 1975. The B cell specificity repertoire, its relationship to definable subpopulations. *Transplant. Rev.* 24:41.
 50. Thompson, M. A., and M. P. Cancro. 1982. Dynamics of B cell repertoire formation: normal patterns of clonal turnover are altered by ligand interaction. *J. Immunol.* 129:2372.
 51. Thompson, M. A., S. Raychaudhuri, and M. P. Cancro. 1983. Restricted adult clonal profiles induced by neonatal immunization: influence of suppressor T cells. *J. Exp. Med.* 158:112.
 52. Kiyono, H., M. D. Cooper, J. F. Kearney, L. M. Mosteller, S. M. Michalek, W. J. Koopman, and J. R. McGhee. 1984. Isotype specificity of helper T cell clones. Peyer's patch Th cells preferentially collaborate with mature IgA B cells for IgA responses. *J. Exp. Med.* 159:798.
 53. Saxen, H., O. Makela, and S. B. Svenson. 1984. Isotype of protective anti-*Salmonella* antibodies in experimental mouse salmonellosis. *Infect. Immun.* 44:633.
 54. Colwell, D. E., S. M. Michalek, D. E. Briles, E. Jirillo, and J. B. McGhee. 1984. Monoclonal antibodies to *Salmonella* lipopolysaccharide: anti-O-polysaccharide antibodies protect C3H mice against challenge with virulent *Salmonella typhimurium*. *J. Immunol.* 133:950.
 55. Pierce, S. K., M. P. Cancro, and N. R. Klinman. 1978. The helper function of individual antigen specific T lymphocytes in enabling the expression of multiple antibody isotypes. *J. Exp. Med.* 148:759.
 56. Virelizier, J. L., R. Postlethwaite, G. C. Schild, and A. C. Allison. 1974. Antibody responses to antigenic determinants of influenza virus hemagglutinin. I. Thymus

- dependence of antibody formation and thymus independence of immunological memory. *J. Exp. Med.* 140:1559.
57. Germanier, R. 1970. Immunity in experimental salmonellosis. I. Protection induced by rough mutants of *Salmonella typhimurium*. *Infect. Immun.* 2:309.
 58. Germanier, R. 1972. Immunity in experimental salmonellosis. III. Comparative immunization with viable and heat-inactivated cells of *Salmonella typhimurium*. *Infect. Immun.* 5:792.
 59. Roantree, R. J. 1967. Salmonella O antigens and virulence. *Annu. Rev. Microbiol.* 21:443.
 60. Ornellas, E. P., R. J. Roantree, and J. P. Steward. 1970. The specificity and importance of humoral antibody in the protection of mice against intraperitoneal challenge with complement-sensitive and complement-resistant Salmonella. *J. Infect. Dis.* 121:113.
 61. Eisenstein, T. K. 1975. Evidence for O antigens as the antigenic determinants in "ribosomal" vaccines prepared from *Salmonella*. *Infect. Immun.* 12:364.
 62. Kiefer, W., P. Gransow, G. Schmidt, and O. Westphal. 1976. Salmonellosis in mice: immunization experiments with *Salmonella-Escherichia coli* hybrids. *Infect. Immun.* 13:1517.
 63. Gerhard, W., T. J. Braciale, and N. R. Klinman. 1975. The analysis of the monoclonal immune response to influenza virus. I. Production of monoclonal anti-viral antibodies *in vitro*. *Eur. J. Immunol.* 5:720.
 64. Klinman, N. R. 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. *J. Exp. Med.* 136:241.
 65. Luderitz, O., A. M. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. *Bacteriol. Rev.* 30:192.
 66. Duran, L. W., and E. S. Metcalf. 1987. Antibody-defective, genetically susceptible CBA/N mice have an altered *Salmonella typhimurium*-specific B cell repertoire. *J. Exp. Med.* 165:29.