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NOTES

Specificity of Monoclonal Antibodies Elicited by Mucosal Infection of BALB/c Mice with Virulent *Shigella flexneri* 2a

ANTOINETTE B. HARTMAN,[1]* LILLIAN L. VAN DE VERG,[1]† CHARLES R. MAINHART,[2] BEN D. TALL,[3] AND SANDRA J. SMITH-GILL[2]

Department of Enteric Infections, Walter Reed Army Institute of Research, Washington, DC 20307-5100[1]; Laboratory of Genetics, National Institutes of Health, Bethesda, Maryland 20892-4255[2]; and Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC 20204[3]

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Protective immunity against shigellosis is thought to be determined by the O-antigen side chains of the lipopolysaccharide (LPS) molecule. To study possible common protective epitopes, monoclonal antibodies reacting with *Shigella flexneri* 2a LPS were generated from BALB/c mice infected ocularly with the virulent serotype 2a strain *S. flexneri* 2457T and tested against a panel of *S. flexneri* LPSs by enzyme-linked immunosorbent and immunoblot assays. Four monoclonal antibodies were identified, all of which showed restricted specificity patterns. Three different patterns of reactivity to LPS possessing the 3,4 group antigen were seen: (i) 2a only, (ii) 2a and 5a, and (iii) 2a, 4a, 5a, and Y. These results have implications for designing a *Shigella* vaccine that will be protective against related serotypes. Electron microscopy studies showed that the monoclonal antibodies bind to the bacterial surface in a patchy pattern, suggesting their potential use for examining the LPS distribution on the surface of the bacteria.

Studies of protective immunity following infection or immunization of humans and monkeys have shown that protection against shigellosis is serotype specific (2, 10, 16). Serotype specificity in *Shigella flexneri* is determined by the structures of the O-antigen side chains of the lipopolysaccharide (LPS) molecule (9, 17). Serotypes 1 to 5, X, and Y (but not serotype 6) have a basic O-antigen chain consisting of a tetrasaccharide repeating unit that is identical to the unsubstituted repeat chain found in *S. flexneri* serotype Y, which is defined as having group 3 and 4 antigen specificity. Other serotype specificities are determined by the addition of α-D-glucosyl and/or *O*-acetyl residues to this basic tetrasaccharide repeating unit (7, 17). These residues confer type (type I to VI) and group (group 3,4; 4; 6; and 7,8) specificities to the basic side chain. If protective immunity against shigellosis is determined by the O antigen, investigation of the antigenic determinants involved in this specificity is important to understanding whether the immune response is directed toward common *S. flexneri* epitopes or to type- or group-specific epitopes. Such studies are also crucial to the development of vaccines against shigellae, since the identification of common protective epitopes would facilitate the design of vaccines that are protective against more than one serotype. Recent studies have demonstrated the presence of cross-reactive antibodies in the sera of humans and animals following natural infection (33). These findings support the concept of vaccine strains that can protect against closely related serotypes.

Both O-antigen-specific and core-specific monoclonal antibodies against *S. flexneri* LPS have been generated in mice and rats by intraperitoneal (i.p.) immunization with heat-killed bacteria. These antibodies have been very useful for the typing of specimens and for immunochemical analyses of type and group antigens (3–6). Since shigellae invade mucosal surfaces, antibodies elicited by mucosal immunization might prove to be more useful in the study of epitopes involved in protection. Here we report the production of anti-LPS monoclonal antibodies following mucosal infection of BALB/c mice with live virulent *S. flexneri* serotype 2a and the characterization of the binding specificities of these antibodies.

It has previously been shown that mice can be used in the Sereny test (21, 28). In this test, the eyes of the animals are inoculated with virulent shigellae which invade the conjunctiva and spread intra- and intercellularly, resulting in the development of keratoconjunctivitis. BALB/c mice were found to be highly susceptible to eye infection with shigellae, although the resulting infection was not as pronounced as that found in guinea pigs. Since the epithelium of the eye is a mucosal surface, we used ocular infection of BALB/c mice for our immunization regimen. Mice were inoculated in each eye with 6.25×10^8 CFU of the virulent serotype 2a strain *S. flexneri* 2457T in normal saline by one of the following two regimens: (i) five immunizations on days 0, 14, 28, 42, and 63 or (ii) two immunizations on days 0 and 22. In both cases, lymphocytes from pooled spleens (two animals) were harvested and fused 3 days after the last immunization with azaguanine-resistant SP2/0-Ag14 cells in a procedure for hybridoma production described previously (30) and were dispensed in 96-well plates at 2×10^5 cells per well. All colonies were initially screened for immunoglobulin secretion by particle concentration fluorescence immunoassays (PCFIAs) with an automated system (Pandex Screen Machine; IDEXX Laboratories, Inc., Westbrook, Maine) as described previously (13). The hybridoma culture supernatants positive for secretion were then screened

* Corresponding author. Mailing address: Department of Enteric Infections, Walter Reed Army Institute of Research, Washington, DC 20307-5100. Phone: (202) 782-3792. Fax: (202) 782-0748. Electronic mail address: dr._antoinette_hartman@wrsmtp-ccmail.army.mil.

† Present address: Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, DC 20307-5100.

for reactivity to *S. flexneri* 2a LPS by enzyme-linked immunosorbent assay (ELISA). Briefly, the test wells of polyvinyl 96-well microtiter plates (Dynatech Laboratories, Alexandria, Va.) were coated with 0.5 μg of *S. flexneri* 2a LPS antigen, prepared by the method of Westphal and Jann (34), in 50 μl of coating buffer (20 mM Na_2CO_3 [pH 9.6]). LPS-coated rows were alternated with rows coated with carbonate buffer alone to obtain background readings. After the plates were washed and blocked with casein filler (20 g of casein, 2.0 g of sodium azide per liter of phosphate-buffered saline [PBS; pH 7.4]), 50 μl of undiluted culture supernatants was added to paired test and blank wells, and the plates were incubated at 37°C for 2 h. Bound antibodies were detected by incubation with a 1:500 dilution of a mixture of anti-mouse immunoglobulin G (IgG), IgA, and IgM conjugated to alkaline phosphatase (Kirkegaard & Perry, Gaithersburg, Md.) followed by development with *p*-nitrophenyl phosphate substrate (Kirkegaard & Perry). The optical densities (ODs) at 405 and 570 nm were read, and clones with OD readings 0.1 unit or more above that of the background wells were then subcloned by the endpoint dilution method (20) three times and were retested for LPS binding. Hybridoma supernatants were also tested for binding to hen egg white lysozyme (HEL) and bovine serum albumin (BSA) by PCFIA as described previously (13) to determine if IgM antibodies were polyspecific and bound proteins or were specific for LPS.

Monoclonal antibodies reacting with *S. flexneri* 2a LPS were only obtained from the fusion of spleen cells from mice immunized two times 22 days apart. This regimen produced 25 secreting hybridomas, 4 of which reacted with *S. flexneri* 2a LPS (Fig. 1). These antibodies were designated antibody HYSF2a1 (IgG2b) and antibodies HYSF2a2, HYSF2a3, and HYSF2a4 (IgM). No reactivity with either BSA or HEL was detected with any of the monoclonal antibodies. In contrast, the fusion of spleen cells from animals immunized five times produced only six secreting colonies, none of which reacted with *S. flexneri* 2a LPS. These results suggest that minimal immunization is more effective for producing LPS-specific antibodies via the mucosal route. The results obtained with this regimen are in agreement with those obtained with the regimen used in a previous work showing that i.p. immunization with heat-killed bacteria given on days 0 and 21 successfully produced LPS-specific hybridomas (3–6).

ELISA endpoint titration and Western immunoblot analysis were used to determine the specificities of the subcloned hybridomas. LPS was prepared from the following strains: *S. flexneri* Nakamura, serotype 1a (Walter Reed Army Institute of Research [WRAIR]); *S. flexneri* Israel 14, serotype 1b (WRAIR), *S. flexneri* 2457T, serotype 2a (WRAIR); *S. flexneri* S041, serotype 2b (WRAIR); *S. flexneri* Tap 3, serotype 3a (WRAIR); *S. flexneri* LB, serotype 3b (WRAIR); *S. flexneri* 4a-25, serotype 4a (WRAIR); *S. flexneri* M76-39, serotype 4b (WRAIR); *S. flexneri* M90T, serotype 5a (WRAIR); *S. flexneri* SFL1, serotype Y (A. Lindberg), *S. flexneri* PE576, serotype X (R. Morona), and *Plesiomonas shigelloides* 5 (WRAIR), the O antigen of which is immunologically identical to that of *Shigella sonnei* (29). Test and blank wells were coated with 0.5 μg of LPS in 50 μl of coating buffer. The antibody concentrations in the supernatants were adjusted to 2 μg/μl, and the supernatants were serially diluted twofold. Endpoint titers were defined as the last dilution with an OD 0.1 unit or greater above that of the background wells. Specificities, including any reaction to the core of the LPS, were also checked by Western immunoblot analysis. A total of 20 μl of LPS stock (10 mg/ml) and 80 μl of water were mixed with 100 μl of 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (4% SDS, 8% 2-β-mercaptoethanol, 0.05% bromophenol blue, 2 M Tris [pH 6.8], 20% glycerol), and the mixture was boiled for 10 min. The mixture was then treated for 1 h at 60°C with 40 μl of proteinase K (2.5 mg/ml; Sigma Chemical Co., St. Louis, Mo.). LPS samples of 8 μl were separated by SDS-PAGE using a 12.5% gel, blotted onto nitrocellulose paper, and incubated with antibody supernatants diluted to 2 μg/μl. After washing, the blots were incubated with anti-mouse IgG or IgM conjugated to alkaline phosphatase (Kirkegaard & Perry) and developed with naphthol AS-MX and fast red (Sigma Chemical Co.).

ELISA endpoint titrations are provided in Fig. 1, and the results of immunoblot analysis are provided in Fig. 2. All four monoclonal antibodies showed restricted specificity patterns, and three different specificity patterns were observed. HySF2a2 showed the most restricted specificity, reacting only with *S. flexneri* 2a LPS. HySF2a1 and HySF2a3 reacted only with 2a and 5a LPSs, while HySF2a4 reacted with 2a, 4a, 5a, and Y LPSs. In the immunoblot analysis, all antibodies gave a typical ladder-like pattern with the same patterns of specificity to LPS that were observed in the ELISA. No reactivity was seen with the core region, which appears as a band at the bottom of the SDS-polyacrylamide gel that reacts with a core-specific monoclonal antibody (5), demonstrating that the specificity is directed toward the O antigen. This is in contrast to the reactivity patterns previously seen in immunoblot analysis with immune serum from guinea pigs infected with *S. flexneri* 2a, in which reactivity was seen with the entire LPS ladder, including the core band (data not shown). Since the LPS samples used in the immunoblot analysis were treated with proteinase K, these results also indicated that the reactivity is not directed against a protein contaminant.

The chemical structures and classifications of the type and group antigens of the *S. flexneri* serotypes used in the present study are provided in Table 1 (19). *S. flexneri* 2a expresses type II and group 3,4 antigens. The 3,4 group antigen, which was defined by absorption data with polyvalent rabbit antisera (9), is also found in serotypes 3b (which also expresses the group 6 antigen), 4a, 5a, and Y, while 1a expresses only the group 4 antigen. Attempts have been made to define the particular epitopes of the 3,4 group antigen by using monoclonal antibodies produced by i.p. immunization with heat-killed *S. flexneri* serotype Y. These studies showed that more than two epitopes can be identified, suggesting that perhaps these epitopes may be exposed or formed by a specific structural conformation(s) and not determined by a particular residue (3). In the present study, all of the monoclonal antibodies elicited by invasion of a mucosal surface recognized LPS expressing the group 3,4 antigen, but did not recognize LPS expressing either the group 4 antigen only (serotype 1a) or group antigens 3, 4, and 6 (serotype 3b), suggesting that the monoclonal antibodies may recognize the group 3 antigen or a conformation encompassing both group antigens. The monoclonal antibodies also did not recognize the serotype 2b LPS, indicating that the type II antigen is not the dominant epitope recognized by these antibodies. Since the reactivity patterns of the monoclonal antibodies vary, more than one epitope is likely being recognized, in agreement with the finding that more than two group 3,4 epitopes can be identified. The three antibodies reacting with 2a and/or 5a LPSs are likely recognizing an epitope influenced by the presence of a single α-D-glycosyl residue on one of the rhamnose residues, while the reactivity of HySf2a4 is directed more toward specificities conferred by the 3,4 group antigen(s). In all cases the presence of the group 6 antigen in serotype 3b or the absence of the group 3 antigen in serotype 1a abrogates the epitope required for

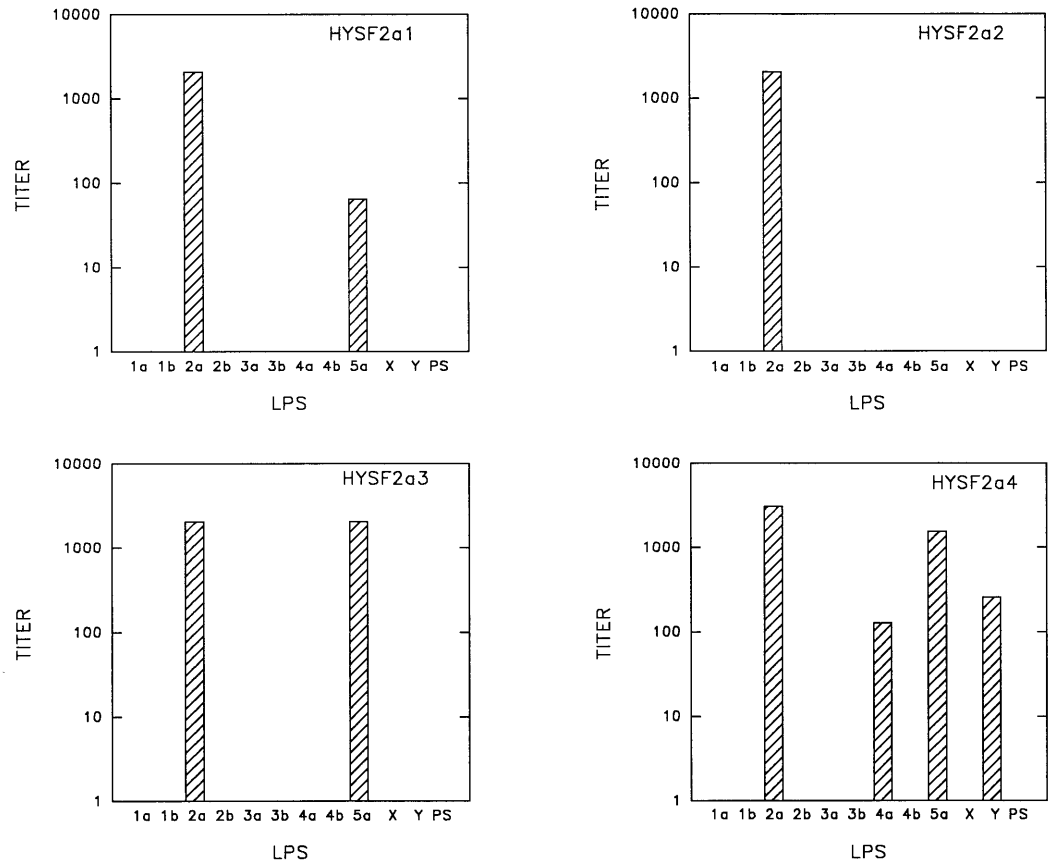

FIG. 1. ELISA endpoint titration of supernatants from all four hybridomas showing reactivity profiles against a panel of *S. flexneri* LPSs. The antibody concentrations in the supernatants were adjusted to 2 μg/ml. Specificities against *S. flexneri* serotype 1a to 5a, X, and Y and *P. shigelloides* (PS) LPSs were tested.

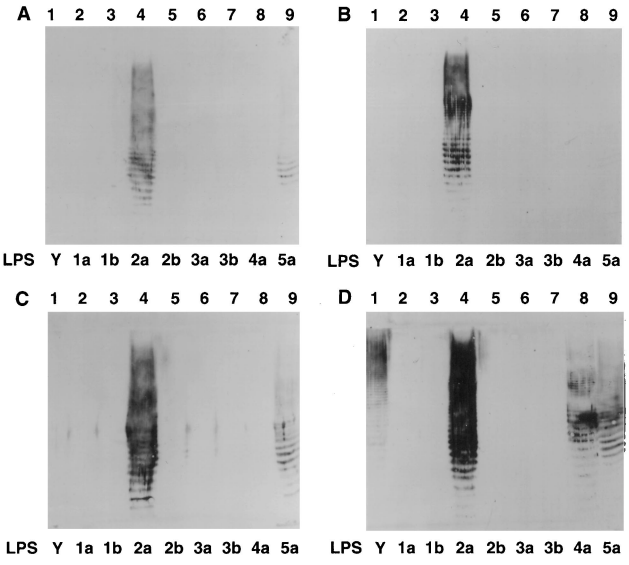

FIG. 2. Immunoblot analysis of reactivities of supernatants against a panel of *S. flexneri* LPSs. (A) HySF2a1; (B) HySF2a2; (C) HySF2a3; (D) HySf2a4. Reactivities against *S. flexneri* serotype X and 4b LPSs and *P. shigelloides* LPS were also tested and were found to be negative (data not shown). There was no reactivity to the core region of the LPS, which would appear as a band at the bottom of the gel (5).

binding. Examples of changes in group antigen specificity by the addition of a single residue are found in serotypes 1a and 1b and serotypes 4a and 4b, in which the addition of the group 6 antigen abrogates the group 4 and group 3,4 antigen specificities, respectively, and in serotypes 2a and 2b and serotypes 5a and 5b, in which the addition of the 7,8 group antigen also abrogates the group 3,4 specificity.

Previously, i.p. immunization with heat-killed bacteria gave 13 monoclonal antibodies recognizing the 2a serotype, 9 of which reacted only with the serotype 2a LPS (4). We found that the serum antibody response to serotype 2a LPS in humans and guinea pigs following natural infection with virulent serotype 2a shigellae or immunization with an attenuated hybrid *Escherichia coli–S. flexneri* 2a vaccine strain EcSf2a-2 (22) shows cross-reactivity with other 3,4 group LPS serotypes, as well as with serotypes 1a and 2b (33). These results and the binding specificities shown by the mucosally derived monoclonal antibodies suggest that mucosal immunization or infection with *S. flexneri* 2a produces a variety of antigenic specificities, with the response to the 3,4 group antigen being the most prevalent one. These findings may be useful in designing a *Shigella* vaccine that will be protective against related serotypes.

An additional application of these antibodies used their antigenic specificities to study the ultrastructural surface distribution of LPSs on the surface of the bacteria. Immunolabeling and electron microscopy of *S. flexneri* 2457T, serotype 2a, and SFL15, serotype Y (A. Lindberg), were carried out with HySF2a1 and HySF2a2, which do not recognize serotype Y

TABLE 1. Type and group designations and chemical structures of each *S. flexneri* serotype used in the study[a]

Serotype	Type	Group	Structure[b]
1a	I	4	-R--R--R--NAG- G^4
1b	I	6	-R--R--R--NAG- A G^4
2a	II	3,4	-R--R--R--NAG- G^4
2b	II	7,8	-R--R--R--NAG- G^3 G^4
3a	III	6,7,8	-R--R--R--NAG- G^3 A
3b	III	6,3,4	-R--R--R--NAG- A
4a	IV	3,4	-R--R--R--NAG- G^6
4b	IV	6	-R--R--R--NAG- A G^6
5a	V	3,4	-R--R--R--NAG- G^3
X	—[c]	7,8	-R--R--R--NAG- G^3
Y	—	3,4	-R--R--R--NAG-

[a] The *S. flexneri* serotypes have been described previously (19).
[b] -R--R--R--NAG-, repeating tetrasaccharide subunit (three rhamnoses plus *N*-acetylglucosamine); A, *O*-acetyl group; G^4, D-glucose, α1-4 linkage; G^3, D-glucose, α1-3 linkage; G^6, D-glucose, α1-6 linkage.
[c] —, no type antigen.

LPS in ELISA and immunoblot analysis. Two immunogold labeling procedures were used. The first procedure involved labeling of the strains with monoclonal antibody supernatant prior to negative staining with sodium phosphotungstate, while the second procedure involved labeling of the strains prior to fixation and processing for ultramicrotomy. For negative staining, the method described by Tall and Nauman (32) was used. Briefly, 20 μl of each bacterial cell suspension in PBS (pH 7.2) was placed on Formvar carbon-coated 300-mesh grids and was allowed to partially dry for 2 to 3 min. The excess liquid was removed, and the grids were immediately placed specimen side down on drops of hybridoma supernatant or PBS-BSA-T20 buffer (PBS containing 1% BSA and 0.1% Tween 20) for 30 min. After thorough washing in buffer, the grids were placed specimen side down onto drops of gold-labeled goat anti-mouse serum of the appropriate isotype (IgG, 10-nm gold particles; IgM, 15-nm gold particles) for 15 min. After washing again with buffer and then with distilled water, the grids were negatively stained with 1% sodium phosphotungstic acid (pH 7.2) and were examined directly with a Philips 400 transmission electron microscope operated at an accelerating voltage of 80 kV. Control cells, which were reacted only with gold conjugate in buffer to determine if nonspecific secondary antibody binding occurred, were all negative. Cells prepared for ultramicrotomy were reacted in 1.5-ml centrifuge tubes with dilutions of monoclonal antibodies made in the PBS-BSA-T20 buffer (a 1:50 dilution appeared to be optimal). After washing three times in the buffer, the cells were prefixed in 3% glutaraldehyde in 0.1 M sodium cacodylate (SCB; pH 7.2) for 1 h at room temperature. The cells were washed three times with SCB and postfixed in 2% OsO_4 in SCB for 1 h at room temperature. The samples were again washed in SCB, dehydrated in a graded series of ethanol (30 to 100%), followed by two exchanges with propylene oxide, and then embedded into EPON (SPI812; Structure Probe Inc., West Chester, Pa.). Ultrathin sections were cut on a Leica Ultracut S ultramicrotome (Leica Inc., Deerfield, Ill.), stained with uranyl acetate and lead citrate according to the method of Reynolds (26), and examined as described above.

As seen in Fig. 3, both HySF2a1 and HySF2a2 bound to the surfaces of *S. flexneri* 2a cells (Fig. 3A and C) but not to the surfaces of cells of *S. flexneri* SFL15, the *S. flexneri* Y serotype strain used as a control (Fig. 3B and D). As seen in Fig. 3A, negative staining showed a highly convoluted exterior cell surface, typical of the fine structure seen for gram-negative bacteria examined by this technique. Thin sectioning (Fig. 3B, C, and D) also showed a typical gram-negative cell wall morphology, namely, a distinct peptidoglycan layer internal to an extensive outer membrane layer. Particularly striking was the localized patchy binding pattern of labeling observed by the negative staining method (Fig. 3A) with HySF2a1, suggesting that expression of the epitope may be at specific cell wall sites. This hypothesis was further supported by the labeling pattern obtained for cells processed by the ultramicrotomy method showing the same patchy labeling pattern extending from the cell surface (Fig. 3C). These results suggest that the sites of epitope expression on the bacteria were localized. Little is known about the distribution of the O side chain antigens on the surfaces of *Shigella* cells. However, it is known that in gram-negative bacteria, after LPS synthesis is completed on the cytoplasmic surface of the inner membrane, the molecules are transferred to the outer membrane at zones of adhesion between the two membranes (1). When gram-negative cells are fixed in a hypertonic medium and examined by electron microscopy, several hundred sites of adhesion between the outer and inner membranes are observed (18). The LPS molecules formed on the inner membrane apparently diffuse to the outer membrane at these sites of adhesion and then spread over the entire membrane. The patchy pattern observed in Fig. 3A may reflect antibody binding to these adhesion zones. It has also been postulated that other localized molecules in the outer membrane, such as phospholipids, may also affect the LPS distribution, creating specific sites such as phage or bacteriocin receptors (27). The role of LPS in pathogenesis has been defined by experiments with rough strains which retain the ability to invade tissue culture cells and multiply intracellularly but which are unable to spread to contiguous cells or to form plaques in tissue culture monolayers (23, 24). Other molecules are also necessary for bacterial intracellular multiplication and intercellular spread. Examples include IpaB, which is involved in lysis of the phagocytic vacuole prior to intracellular multiplication; VirG (IcsA), which is necessary for intracellular movement and intercellular spread; and IcsB, which is required for lysis of the two membranes of the protrusion containing a bacterium, thus releasing it into the contiguous cell (11). Additional studies must be done to examine whether the distribution of LPS on the surface of the bacteria plays a role in the expression of virulence in shigellae.

The importance of antibodies against the O antigen to the development of protective immunity has been shown previously (8, 10, 16, 25, 31). However, it is important for the design of multivalent *Shigella* vaccines to determine any cross protection against closely related serotypes. The results from the

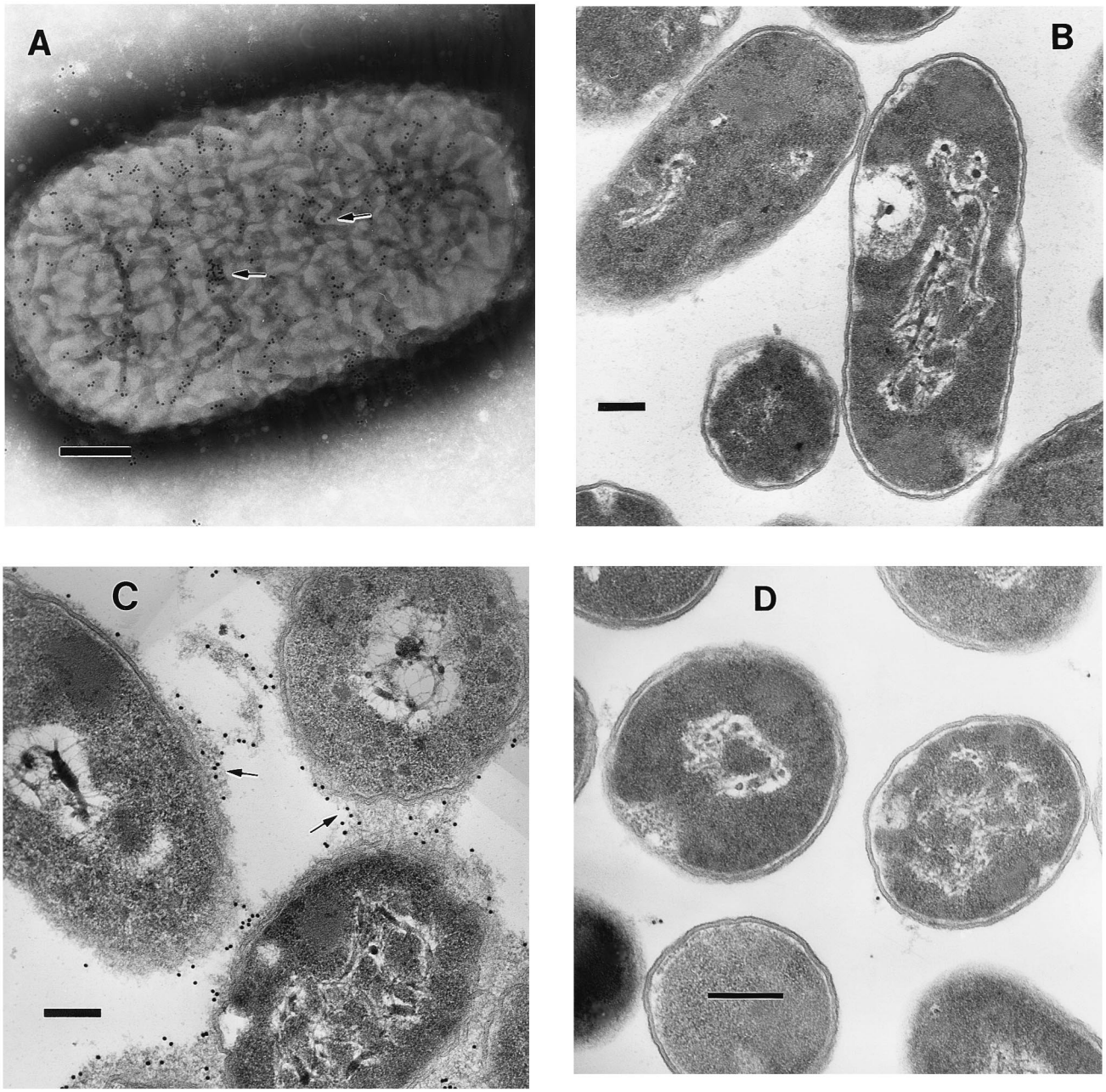

FIG. 3. Transmission electron photomicrographs of *S. flexneri* 2457T, serotype 2a, and control *Shigella flexneri* SFL15, serotype Y, labeled with HySF2a1 or HySF2a2 and goat anti-mouse IgG or IgM conjugated to colloidal gold. Note the patchy binding pattern of labeling of the cell surface (arrows). (A) Negative staining of *S. flexneri* 2a with HySF2a1 followed by reaction with a 1:10 dilution of goat anti-mouse IgG conjugated to colloidal gold (10 nm). (B) Immunogold staining by ultramicrotomy of *S. flexneri* Y with HySF2a1 followed by reaction with a 1:10 dilution of goat anti-mouse IgG conjugated to colloidal gold. (C) Immunogold staining by ultramicrotomy of *S. flexneri* 2a with HySF2a2 followed by reaction with a 1:10 dilution of goat anti-mouse IgM conjugated to colloidal gold (15 nm). (D) Immunogold staining by ultramicrotomy of *S. flexneri* Y with HySF2a2 followed by reaction with a 1:10 dilution of goat anti-mouse IgM conjugated to colloidal gold. Bars, 0.25 μm.

present study and the serum cross-reactivity found in humans and animals immunized or infected with serotype 2a (33) suggest that an *S. flexneri* 2a vaccine might also protect against other serotypes expressing the 3,4 group antigen. Data from studies that have used the guinea pig keratoconjunctivitis model (14, 15, 28) have demonstrated that infection with a serotype 2a strain can protect against challenge with a virulent Y strain (12). Further studies on the cross-reactivities of monoclonal antibodies and the serum antibody responses of animals and humans generated by immunization or infection by a mucosal route can determine other potential cross-reactivity patterns that might be useful in designing a multivalent vaccine.

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REFERENCES

1. **Bayer, M. E.** 1968. Areas of adhesion between wall and membrane of *Escherichia coli*. J. Gen. Microbiol. **53:**395–404.
2. **Black, R. E., M. M. Levine, M. L. Clements, G. Losonsky, D. Herrington, S. Berman, and S. B. Formal.** 1987. Prevention of shigellosis by a *Salmonella*

typhi-Shigella sonnei bivalent vaccine. J. Infect. Dis. **155:**1260–1265.
3. **Carlin, N. I. A., D. R. Bundle, and A. A. Lindberg.** 1987. Characterization of five *Shigella flexneri* variant Y-specific monoclonal antibodies using defined saccharides and glycoconjugate antigens. J. Immunol. **138:**4419–4427.
4. **Carlin, N. I. A., and A. A. Lindberg.** 1983. Monoclonal antibodies specific for O-antigenic polysaccharides of *Shigella flexneri*: clones binding to II, II:3,4, and 7,8 epitopes. J. Clin. Microbiol. **53:**110–115.
5. **Carlin, N. I. A., and A. A. Lindberg.** 1986. Monoclonal antibodies specific for *Shigella flexneri* lipopolysaccharides: clones binding to type I and type III: 6,7,8 antigens, group 6 antigen, and a core epitope. Infect. Immun. **53:**103–109.
6. **Carlin, N. I. A., and A. A. Lindberg.** 1987. Monoclonal antibodies specific for *Shigella flexneri* lipopolysaccharides: clones binding to type IV, V, and VI antigens, group 3,4 antigen, and an epitope common to all *Shigella flexneri* and *Shigella dysenteriae* type 1 strains. Infect. Immun. **55:**1412–1420.
7. **Carlin, N. I. A., S. S. Lindberg, K. Bock, and D. R. Bundle.** 1984. The *Shigella flexneri* O-antigenic polysaccharide chain. Nature of the biological repeating unit. Eur. J. Biochem. **139:**189–194.
8. **Cohen, D., M. S. Green, C. Block, R. Slephon, and Y. Lerman.** 1992. Natural immunity to shigellosis in two groups with different previous risks of exposure to *Shigella* is only partly expressed by serum antibodies to lipopolysaccharide. J. Infect. Dis. **165:**785–787.
9. **Ewing, W. H.** 1986. The genus *Shigella*, p. 155–166. *In* P. R. Edwards and W. H. Ewing (ed.), Identification of Enterobacteriaceae. Elsevier Science Publishing, New York.
10. **Formal, S. B., E. V. Oaks, R. E. Olsen, M. Wingfield-Eggleston, P. J. Snoy, and J. P. Cogan.** 1991. Effect of prior infection with virulent *Shigella flexneri* 2a on the resistance of monkeys to subsequent infection with *Shigella sonnei*. J. Infect. Dis. **164:**533–537.
11. **Goldberg, M. B., and P. J. Sansonetti.** 1993. *Shigella* subversion of the cellular cytoskeleton: a strategy for epithelial colonization. Infect. Immun. **61:**4941–4946.
12. **Hartman, A. B.** Unpublished data.
13. **Hartman, A. B., C. P. Mallett, J. Srinivasappa, B. S. Prabhakar, A. L. Notkins, and S. J. Smith-Gill.** 1989. Organ reactive autoantibodies from non-immunized BALB/c mice are polyreactive and express non-biased V_H gene usage. Mol. Immunol. **26:**359–370.
14. **Hartman, A. B., C. J. Powell, C. L. Schultz, E. V. Oaks, and K. H. Eckels.** 1991. Small-animal model to measure efficacy and immunogenicity of *Shigella* vaccine strains. Infect. Immun. **59:**4075–4083.
15. **Hartman, A. B., L. L. Van De Verg, H. H. Collins, Jr., D. B. Tang, N. O. Bendiuk, D. N. Taylor, and C. J. Powell.** 1994. Local immune response and protection in the guinea pig keratoconjunctivitis model following immunization with *Shigella* vaccines. Infect. Immun. **62:**412–420.
16. **Herrington, D. A., L. Van De Verg, S. B. Formal, T. L. Hale, B. D. Tall, S. J. Cryz, E. C. Tramont, and M. M. Levine.** 1990. Studies in volunteers to evaluate candidate *Shigella* vaccines: further experience with a bivalent *Salmonella typhi-Shigella sonnei* vaccine and protection conferred by previous *Shigella sonnei* disease. Vaccine **8:**353–357.
17. **Kenne, L., B. Lindberg, K. Petersson, E. Katsenellenbogen, and E. Romanowska.** 1978. Structural studies of *Shigella flexneri* O-antigens. Eur. J. Biochem. **91:**279–284.
18. **Lieve, L. L., and B. D. Davis.** 1980. Cell envelope; spores, p. 82–88. *In* B. D. Davis, R. Dulbecco, H. N. Eisen, and H. S. Ginsberg (ed.), Microbiology: including immunology and molecular genetics, 3rd edition. Harper & Row, Publishers, Inc., Hagerstown, Md.
19. **Lindberg, A. A., E. Ekwall, and N. I. A. Carlin.** 1988. *Shigella flexneri* O-antigen specific enzyme immunoassay: lipopolysaccharides and synthetic glycoconjugates as antigen. Serodiagn. Immunother. **2:**63–78.
20. **McKearn, T. J.** 1980. Cloning of hybridoma cells by limiting dilution in fluid phase, p. 374. *In* R. H. Kennett, T. J. McKearn, and K. B. Bechtol (ed.), Monoclonal antibodies: hybridomas: a new dimension in biological analyses. Plenum Press, New York.
21. **Murayama, S. Y., T. Sakai, S. Makino, T. Kurata, C. Sasakawa, and M. Yoshikawa.** 1986. The use of mice in the Sereny test as a virulence assay of shigellae and enteroinvasive *Escherichia coli*. Infect. Immun. **51:**696–698.
22. **Newland, J. W., T. L. Hale, and S. B. Formal.** 1992. Genotypic and phenotypic characterization of an *aroD* deletion-attenuated *Escherichia coli* K12-*Shigella flexneri* 2a hybrid expressing *Shigella flexneri* 2a somatic antigen. Vaccine **10:**766–776.
23. **Okamura, N., and R. Nakaya.** 1977. Rough mutants of *Shigella flexneri* 2a that penetrate tissue culture cells but do not evoke keratoconjunctivitis in guinea pigs. Infect. Immun. **17:**4–8.
24. **Okamura, N., R. Nakaya, S. Kondo, M. Murakami, and K. Hisatsune.** 1983. HeLa cell invasiveness and O antigen of *Shigella flexneri* as separate and prerequisite attributes of virulence to evoke keratoconjunctivitis in guinea pigs. Infect. Immun. **39:**505–513.
25. **Phalipon, A., P. Michetti, M. Kaufman, J. M. Cavaillon, M. Huerre, J. P. Kraehenbuhl, and P. J. Sansonetti.** 1994. Protection against invasion of the mouse pulmonary epithelium by a monoclonal IgA directed against *Shigella flexneri* lipopolysaccharide. Ann. N. Y. Acad. Sci. **730:**356–358.
26. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17:**298–342.
27. **Rothfield, L., and D. Romeo.** 1971. Enzyme reactions in biological membranes, p. 251–284. *In* L. Rothfield (ed.), Structure and function of biological membranes. Academic Press, Inc., New York.
28. **Sereny, B.** 1957. Experimental keratoconjunctivitis shigellosa. Acta Microbiol. Acad. Sci. Hung. **4:**367–376.
29. **Shimada, T., and R. Sakazaki.** 1978. On the serology of Plesiomonas shigelloides. Jpn. J. Med. Sci. Biol. **31:**135–142.
30. **Smith-Gill, S. J., A. C. Wilson, M. Potter, E. M. Prager, R. J. Feldmann, and C. R. Mainhart.** 1982. Mapping the antigenic epitope for a monoclonal antibody against lysozyme. J. Immunol. **128:**314–322.
31. **Tacket, C. O., S. B. Binion, E. Bostwick, G. Losonsky, M. J. Roy, and R. Edelman.** 1992. Efficacy of bovine milk immunoglobulin concentrate in preventing illness after *Shigella flexneri* challenge. Am. J. Trop. Med. Hyg. **47:**276–283.
32. **Tall, B. D., and R. K. Nauman.** 1994. Outer membrane antigens of oral Treponema species. J. Med. Microbiol. **40:**62–69.
33. **Van De Verg, L. L., N. O. Bendiuk, K. Kotloff, M. M. Marsh, J. L. Ruckert, J. L. Puryear, D. N. Taylor, and A. B. Hartman.** Cross-reactivity of *Shigella flexneri* serotype 2a O antigen antibodies following immunization or infection. Vaccine, in press.
34. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide extraction with phenol: water and further application of the procedure. Methods Carbohydr. Chem. **5:**82–91.