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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

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Monoclonal antibodies reactive with *Shigella flexneri* O antigens were generated in both mouse and rat systems. Antibody-producing hybridomas were screened in an enzyme-linked immunosorbent assay using chemically defined lipopolysaccharides as antigens, and the epitope specificities were determined with a panel of lipopolysaccharides and synthetic O-antigen-specific glycoconjugates as antigens. To verify the specificity seen in the enzyme-linked immunosorbent assay, the antibodies were used in agglutination against a large number of *S. flexneri* strains. Monoclonal antibodies with the following specificities were identified: type antigen IV (reactive with serotype 4a and 4b bacteria); type antigen V (reactive with serotype 5a and 5b bacteria); type antigen VI (reactive with serotype 6 bacteria); group antigen 3,4 (reactive with serotype 1a, 2a, 3b, 4a, 5a, and Y bacteria); and group antigen 1 (reactive with an epitope present on all *S. flexneri* and *Shigella dysenteriae* type 1 bacteria). Furthermore, a monoclonal antibody defining a new O-antigenic epitope present on some *S. flexneri* strains of serotypes 4a, X, and Y was characterized (4X). The monoclonal antibodies analyzed in this study define epitopes described by polyclonal antisera (type antigens IV, V, and VI), define a hitherto uncharacterized epitope (group antigen 1), and finally identify new epitopes in what has previously been considered as one epitope (group antigen 3,4 and type antigen IV). These immunochemically characterized monoclonal antibodies may have a powerful potential in studies of the importance of humoral immunity in shigellosis.

Shigella flexneri serotyping is based on serological identification of the O antigens (18). The O antigens are a part of the lipopolysaccharide (LPS) molecule, which is inserted in the outer membrane of gram-negative bacteria. Chemically, the O-antigen is a polysaccharide chain of variable length, built up by repetitive sequences of tetra- to hexasaccharides.

The serological classification of *S. flexneri* is based on the use of rabbit antisera elicited by heat-killed *S. flexneri* bacteria. To render the antisera specific, they are absorbed with heat-killed bacteria carrying cross-reactive O-antigenic epitopes (18). Since there are vast structural similarities between the different serotypes of *S. flexneri*, the resulting absorbed antisera often show residual cross-reactivity or are of lower titer.

The structures of the *S. flexneri* O antigens have been elucidated (29–31). It was found that a basic tetrasaccharide repeating unit consisting of three rhamnoses and one *N*-acetylglucosamine was common to all *S. flexneri* strains (except serotype [17]): →2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→. This structure is identical to the O antigen of *S. flexneri* subserotype Y bacteria. The other type and group antigens are consequences of substitution of this basic tetrasaccharide with D-glucose or *O*-acetyls or both, which add new or mask existing antigenic epitopes (30). Type antigens (roman numerals) are shared between members of the same serotype (e.g., serotypes 1a and 1b); group antigens (arabic numerals) are shared by members of different serotypes (e.g., group antigen 6 is present in serotypes 1b, 3a, 3b, and 4b (Fig. 1). Comparative analyses of the structure of the different O antigens have permitted the elucidation of the different moieties responsible for most of the antigenic epitopes of *S. flexneri* (Fig. 1). Recently, we have concluded that the type antigen III is nonexistent and that what has been called type antigen III is just the consequence of the presence of one or two group antigens on the basal repeating unit (12). The only antigen to which no structural entity has been ascribed is the group antigen 3,4.

In this paper we report on the generation of hybridomas secreting antibodies specific for the group antigen(s) 3,4, a hitherto uncharacterized epitope (group antigen 1 common to all *S. flexneri* and to *Shigella dysenteriae* type 1). We also report on antibodies recognizing an epitope in serotype 4 strains, which is provisionally designated 4X (E1037), and on type V- and VI-specific monoclonal antibodies.

MATERIALS AND METHODS

Bacterial strains. *Plesiomonas shigelloides* (NBL strain 650) and *S. flexneri* strains of serotypes 1a, 1b, 2a, 2b, 3a, 4a, 4b, 5b, X, Y, and 6; 4bR, a rough mutant with the complete R3 core (28); and *Staphylococcus aureus* (Cowan 1) were from the strain collection at the Department of Bacteriology, National Bacteriological Laboratory, Stockholm, Sweden. *S. flexneri* serotype 3b (a bacteriophage Sf6-lysogenized subserotype Y strain) was available from previous investigations (25, 34). The E1037 4X strain was kindly provided by Bernhard Rowe, Central Public Health Laboratory, Colindale, England. For coagglutination, clinical isolates of *S. flexneri*, as well as *S. dysenteriae* and *S. boydii* type culture

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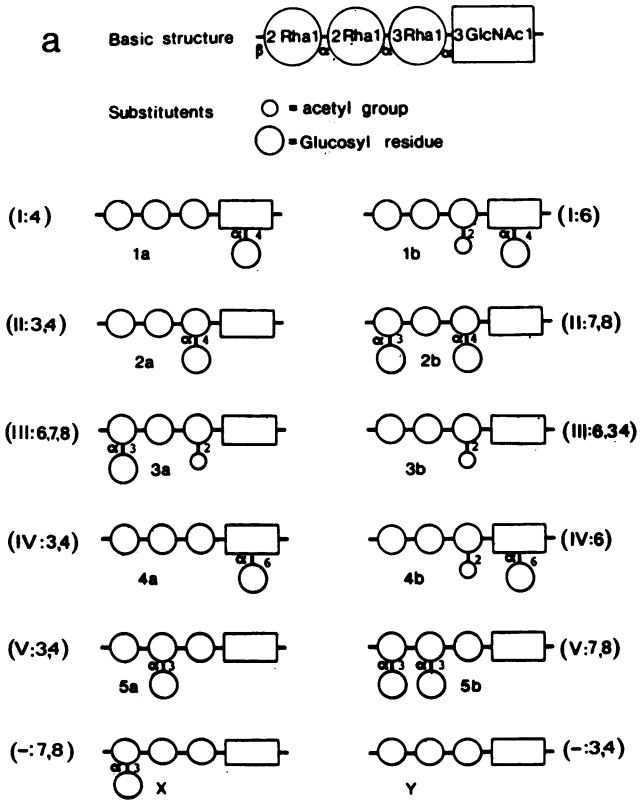

FIG. 1. Chemical structure of (a) *S. flexneri* serotypes 1 through 5, X, and Y; (b) *S. fleneri* type 6; and (c) *S. dysenteriae* type 1 O antigens. Abbreviations: Rha, rhamnose; GlcNAc, *N*-acetylglucosamine; GalA, galacturonic acid; GalNAc, *N*-acetylgalactosamine; Gal, galactose; OAc, *O*-acetyl.

strains from the National Bacteriological Laboratory strain collection, were used.

Preparation of LPS. Individual colonies of bacterial strains selected for LPS production were agglutinated with rabbit anti-*S. flexneri* typing antisera as well as with monoclonal anti-*S. flexneri* reagents developed in this laboratory (8, 10) before fermentor growth. Bacteria were grown in submerged culture, and LPS was extracted by hot phenol-water (47) for smooth bacteria and by phenol-chloroform-light petroleum (23) for rough bacteria. Removal of *O*-acetyls from LPS (de-O-acetylation) was accomplished by boiling in 0.15 M NaOH for 2 h. After neutralization with 1 M HCl and dialysis against distilled water, de-O-acetylated LPSs were lyophilized (12).

The chemical characterization of the rough LPS used has been described elsewhere (27). Smooth *S. flexneri* LPSs were subjected to sugar analysis as described by Sawardeker et al. (42).

For proton nuclear magnetic resonance spectroscopy, a JEOL GX 270 instrument was used. The spectra were recorded for solutions in D_2O at 70 or 85°C with either tetramethylsilane as external standard or H_2O as internal standard.

Cell culture media. The cell culture medium used for standard growth and for hypoxanthine-aminopterin-thymidine selection and growth in serum-free supplemented medium was as described earlier (8, 14).

Immunization of animals. Female BALB/c or C57BL/6 mice were immunized on days 0 (intraperitoneally) and 21 (intravenously) with 10^8 heat-killed *S. flexneri* (day 0 dose suspended in an equal volume of Freund complete adjuvant; day 21 dose suspended in phosphate-buffered saline [PBS]). For immunization of LOU/C rats, the same immunization schedule was followed with the exception that only one individual, of either sex, was used, and the dose of bacteria was 10^9 for each immunization. Fusions were performed 4 days after the last immunization.

Production of hybridomas. The procedure for fusions in mouse cells was essentially that of Köhler and Milstein (32), with modifications from de St. Groth and Scheidegger (15). Approximately 10^8 spleen cells from three pooled spleens of *S. flexneri*-immunized mice were fused with 5×10^7 (hypoxanthine-guanine-phosphoribosyltransferase negative) Sp2/0-Ag 14 cells (43), using polyethylene glycol 4000 (E. Merck AG, Darmstadt, Federal Republic of Germany). For production of rat hybridomas, the washed spleen cells (5×10^7) from one LOU/C rat were fused with the rat myeloma cell line Y3.Ag.1.2.3 (24) in a 1:1 ratio. After fusion, cells were grown in hypoxanthine-aminopterin-thymidine (Sigma Chemical Co., St. Louis, Mo.) selection medium (35) in 96-well microtiter plates.

Screening and selection of hybridomas. Putative hybrids were tested in an enzyme-linked immunosorbant assay (ELISA), using the homologous LPS as antigen. Positive clones ($A_{405} \times 100$ min > 1.0) were further tested against a panel of *S. flexneri* LPSs of different serotypes and wells with only coating buffer and bovine serum albumin (BSA). Hybridomas secreting antibody of the desired specificity were recloned twice or until 100% cloning efficiency was accomplished by limiting dilution.

All cell lines selected in this way were grown as ascites tumors in Pristane (Aldrich Chemical Co., Milwaukee, Wis.)-treated (36) BALB/c mice or LOU/C rats. Ascites fluid was tested in ELISA endpoint titration against a set of 15 different antigens including LPSs from all *S. flexneri* serotypes, rough *S. flexneri* LPS (4bR), and LPS representing *S. sonnei* phase 1 antigen (*P. shigelloides* LPS) (19, 41).

Preparation of sensitized staphylococci and coagglutination. The procedure for preparation of sensitized staphylococci and coagglutination has been described in detail elsewhere (9, 33). Briefly, 0.1 ml of ascites fluid was added to 1.0 ml of 10% formaldehyde and heat-treated staphylococci in 0.1 M $NaPO_4$ buffer (pH 8.0). After incubation and washing, the reagent was made to 2% (vol/vol) in the same buffer. Coagglutination was done with boiled *Shigella* cultures on a slide glass. Agglutination was recorded as ++ when clear as seen by the naked eye, and as + if a magnifying glass was needed for observation.

ELISA. The ELISA procedure has been described in detail earlier (8, 20, 45). Briefly, culture supernatants, undiluted or diluted in PBS–0.05% Tween 20, were added to washed (0.15 M NaCl, 0.05% Tween 20) 96-well flat-bottomed microtiter plates (A/S Nunc, Roskilde, Denmark) that had previously been coated overnight at 20°C with 0.1 ml of LPS (10 μg/ml in 0.05 M carbonate buffer, pH 9.6) followed by 1% BSA in the same buffer. The first antibody incubation (0.1 ml) was left for 4 h at 20°C, plates were

washed as before, and 0.1 ml of an alkaline phosphatase-rabbit anti-mouse immunoglobulin (13) diluted in PBS–0.05% Tween 20 was added. Alternatively, an alkaline phosphatase-goat anti-rabbit immunoglobulin conjugate was used. (Both conjugates detect immunoglobulin G [IgG], IgM, and IgA [data not shown]). For detection of rat antibodies, an alkaline phosphatase-goat anti-rat IgG (whole molecule) conjugate (Sigma) was used. Trays were incubated at 20°C overnight. For developing, plates were washed as before and 0.1 ml of a 1 M diethanolamine–0.5 mM $MgCl_2$ buffer (pH 9.8) containing sodium *p*-nitrophenol phosphate (1 mg/ml) was added. Plates were incubated at 37°C (for detection of mouse antibodies) or at 20°C (for detection of rabbit and rat antibodies). For endpoint titrations, 10-fold dilution steps, from 10^{-2} to 10^{-6} of the serum or ascites to be tested were made in PBS–0.05% Tween 20. Endpoint titers, defined as the reciprocal serum dilution giving $A_{405} \times 100$ min at 20 or 37°C = 0.1, were calculated by linear regression on a PET Commodore CBM 8032 business computer interfaced with a Flow photometer (Flow Laboratories Ltd., Irvine, Scotland), utilizing an ELISA program obtained from Meddata Digital AB, Solna, Sweden.

For ELISA antigen saturation experiments, LPS or de-*O*-acetylated LPS at 50, 10, 1, 0.1, and 0.01 μg/ml was used for coating. After blocking of residual binding sites with BSA, all wells were incubated with one concentration of ascites or serum (10^{-3} to 10^{-5}). Plates were washed and developed as described above. Antibody class and subclasses for mouse monoclonal antibodies were determined by ELISA using serum-free culture supernatants of the antibody-producing cell lines as coating antigen (diluted 1/10 in PBS). The coated wells were then washed as described above and incubated with rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA heavy-chain antisera and rabbit anti-mouse κ and λ lightchain antisera (Litton Bionetics, Kensington, Md.) diluted in PBS–0.05% Tween 20 (1:8,000). Plates were incubated, washed, and developed as described above.

Antibody class and subclass for rat monoclonal antibodies were determined in ELISA with antigen-coated wells using a Zymed Monoab-ID EIA kit (ZyMED Laboratories, Inc., San Francisco, Calif.). The kit contains biotinylated rabbit anti-rat IgG1, IgG2a, IgG2b, IgG2c, IgGA, IgGM, IgE, κ light chain, and peroxidase-conjugated strepavidin.

RESULTS

Production of *S. flexneri* O-antigen-specific hybridomas. (i) *S. flexneri* O-antigen IV-specific monoclonal antibodies. In *S. flexneri* serology, only serotype 4a (type antigen IV, group antigen 3,4) and serotype 4b (type antigen IV, group antigen 6) are recognized (Fig. 1). It has been found, however, that the expression of the group antigen 3,4 in 4a strains is variable (18), and strains which lack the group antigen 3,4 can be isolated (IV:–). The basis of this variability is unknown. Recently, B. Rowe (personal communication) found strains which carry the type antigen IV and the group antigen 7,8. Such strains, hitherto unknown, have been given the provisional designation serotype E1037 (=4X).

Sixteen fusions with *S. flexneri* 4a (whole bacteria or cell envelopes) and nine fusions with *S. flexneri* 4b bacteria in BALB/c or C57BL/6 mice did not yield any hybridoma producing antibodies specific for the type IV antigenic epitope, as present in *S. flexneri* 4a and 4b strains. Approximately 1,200 clones positive by ELISA were tested (positive wells had an $A_{405} \times 100$ min ≥ 1.0; negative wells were generally ≤0.1) In fusions with *S. flexneri* 4a, only antibodies specific for *S. flexneri* 4a and the provisional serotype E1037 (4X), or unspecific antibodies reactive with BSA-coated wells, were detected (MASF IV-1). No antibodies specific for 4b LPS were found. In fusions with serotype 4b bacteria, only antibodies specific for group antigen 6 (binding to LPS of serotypes 1b, 3a, 3b, and 4b) or unspecific antibodies were detected.

Since no hybridomas producing type antigen IV-specific antibodies could be selected after all these attempts, the possibility of a genetically determined unresponsiveness in the mice was raised. We therefore immunized LOU/C rats for fusion experiments. Two fusions with spleen cells from LOU/C rats immunized with two different strains of *S. flexneri* 4a bacteria yielded 9 clones that bound to both *S. flexneri* 4a and 4b LPS and 72 clones that reacted unspecifically. Seven of the specific clones were successfully recloned and used for ascites production (MASF IV-2). One fusion with spleen cells from an *S. flexneri* 4b bacteria-immunized LOU/C rat yielded 20 supernatants reactive with group antigen 6 and 6 supernatants that were unspecific, in analogy with the mouse experiments.

(ii) *S. flexneri* O-antigen V-specific antibodies. A fusion of *S. flexneri* 5b-immunized BALB/c mice with the plasmacytoma cell line Sp2/0 resulted in 28 culture supernatants reactive with type 5b LPS in ELISA ($A_{405} \geq 1.0$). After initial testing against different *S. flexneri* LPSs, four clones were selected with specificity for both 5a and 5b LPS. These were succesfully recloned and used for ascites production.

(iii) *S. flexneri* O-antigen VI-specific monoclonal antibodies. In one fusion with Sp2/0 cells and spleen cells from *S. flexneri* serotype 6-immunized BALB/c mice, 67 culture supernatants scored as positive in an ELISA ($A_{405} \geq 1.0$) using *S. flexneri* type 6 LPS as antigen. After initial screening, 12 clones were discarded as cross-reactive, and 19 were selected for recloning. Twelve of these were successfully recloned and used for ascites production (MASF VI-1).

(iv) *S. flexneri* Y-specific monoclonal antibodies. From five fusions with spleen cells from BALB/c mice, immunized with different *S. flexneri* subserotype Y strains, 30 culture supernatants were screened for reactivity with *S. flexneri* Y LPS ($A_{405} \geq 1.0$). Five of these clones reacted with one or more of the LPSs which contain group antigen 3,4 and also with two glycoconjugates [α-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→2)α-L-Rha*p*-(1→2)-α-L-Rha*p*]$_n$–BSA (n = 1 or 2) obtained by phage-mediated cleavage of the O-polysaccharide chain as described (11). All five clones were recloned and used for ascites production (MASF B and MASF Y-1, Y-2, Y-3, and Y-4).

Spleen cells from a LOU/C rat immunized with *S. flexneri* serotype Y bacteria were fused with the rat myeloma cell line Y3.Ag.1.2.3 (24). Fifteen culture supernatants were scored as positive after initial screening against Y LPS ($A_{405} \geq 1.0$). The reaction pattern of four clones against a test battery of *S. flexneri* LPS indicated a group antigen 3,4 specificity. They were recloned, and three were used for ascites production in Pristane-treated LOU/C rats (MASF Y-5).

ELISA endpoint titration of *S. flexneri* monoclonal mouse and rat antibodies. The immunochemical specificities of the monoclonal antibodies were determined by using ELISA endpoint titrations against a battery of saccharide antigens: well-characterized *S. flexneri* LPS and synthetic glycoconjugates.

***S. flexneri* IV monoclonal antibodies.** All anti-*S. flexneri* type IV monoclonal antibodies developed in the BALB/c and C57BL/6 mice showed identical binding patterns when

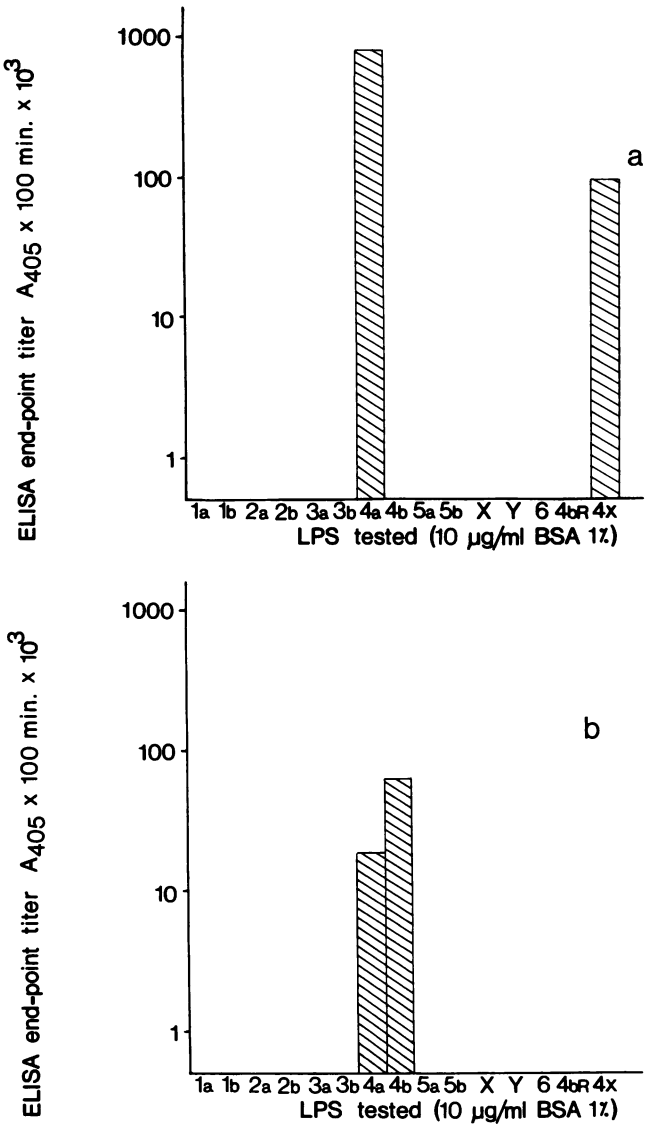

FIG. 2. ELISA endpoint titration of ascitic fluid from two different hybridomas obtained with *S. flexneri* 4a as immunogen. (a) Mouse monoclonal antibody specific for the serotype 4a and 4X antigens, MASF IV-1 (IgG3). (b) Rat monoclonal antibody specific for the serotype 4a and 4b antigens, MASF IV-2 (IgG2c).

tested as ascites fluid in ELISA: high reactivitiy with *S. flexneri* 4a and provisional serotype E1037 (4X) LPS only. One clone (MASF IV-1) of the IgG3 subclass was selected for all further studies (Fig. 2a). The rat monoclonal antibodies were all specific for serotypes 4a and 4b; two were of the IgG2c subclass. The hybridoma in the latter group with the highest titer in ELISA (MASF IV-2) was selected for all further work (Fig. 2b).

***S. flexneri* O-antigen V monoclonal antibodies.** Of the *S. flexneri* serotype 5 specific monoclonal antibodies (MASF V-1 to 4), two were of the IgG3 subclass, one was IgM, and one was IgG1. The IgG3 antibody with the highest titer in ELISA was selected for all further work. The specificity in ELISA (Fig. 3a) for this antibody was exclusive for *S. flexneri* 5a and 5b LPS, indicating that it recognized the α-D-Glc-(1→3)-α-L-rhamnosyl II structure (Fig. 1) as its antigenic epitope. The presence of an α-D-glucose linked α-1,3 to rhamnosyl I (as in 5b) did not interfere with binding of this antibody.

***S. fleneri* O-antigen VI monoclonal antibodies.** The IgG3 antibody with the highest titer in ELISA (MASF VI-1) was chosen from the available *S. flexneri* serotype 6-specific antibodies (Fig. 3b). In ELISA it bound only to *S. flexneri* LPS of serotype 6.

***S. flexneri* Y monoclonal antibodies.** All six monoclonal antibodies originating from the *S. flexneri* Y-primed B-cells reacted differently in ELISA (Table 1). The MASF B antibodies reacted with all *S. flexneri* LPSs tested except 4bR, the rough mutant. These antibodies were also tested in a glycoconjugate ELISA in which different synthetic (5) as well as phage endoglycanase (11) derived oligosaccharides linked to BSA were used as antigens (7). The MASF B antibodies reacted with all antigens tested (Fig. 4). MASF Y-1 bound to 4a and Y LPS, both carrying the group antigen

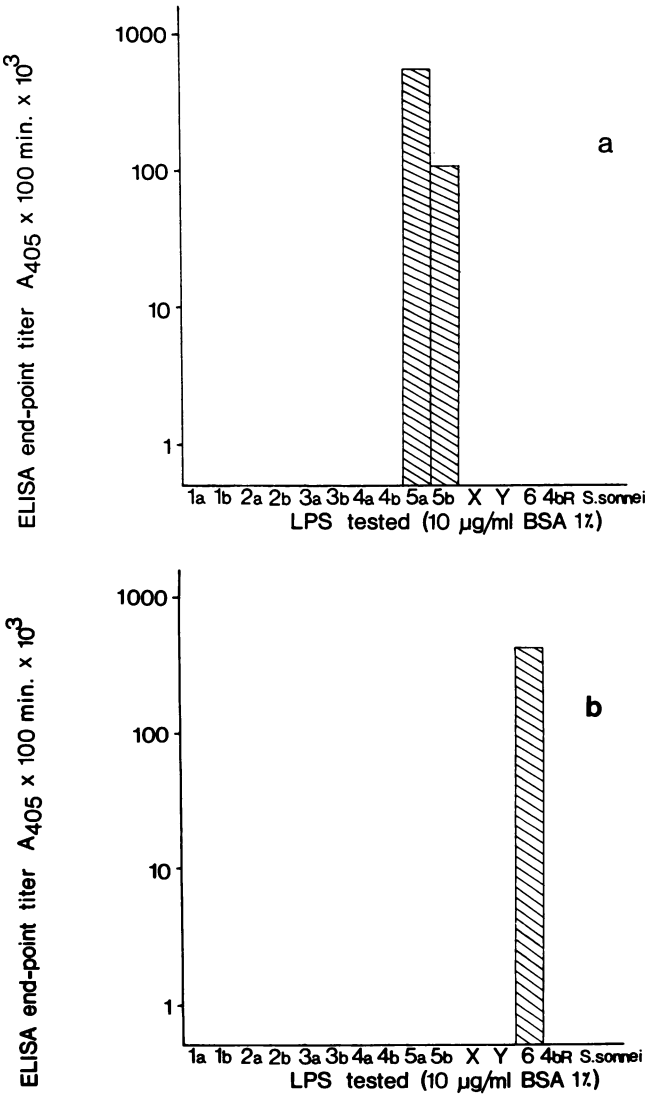

FIG. 3. ELISA endpoint titration of ascitic fluid from one hybridoma obtained with *S. flexneri* 5b as immunogen and one hybridoma obtained with *S. flexneri* 6 as immunogen. (a) Monoclonal antibody specific for the type antigen V, MASF V-1 (IgG3). (b) Monoclonal antibody specific for the type antigen VI, MASF VI-1 (IgG3).

TABLE 1. ELISA endpoint titrations of *S. flexneri* monoclonal antibodies and a rabbit anti-*S. flexneri* Y antiserum against LPS from all *S. flexneri* serotypes

LPS serotype (antigenic formula)	Endpoint titer[a] (10^3) of antibody:						
	MASF B	MASF Y-1	MASF Y-2	MASF Y-3	MASF Y-4	MASF Y-5	Rabbit
1a (I:4)	124	<1	<1	<1	<1	<1	<1
1b (I:6)	110	<1	<1	<1	<1	<1	<1
2a (II:3,4)	160	<1	<1	9	70	125	8
2b (II:7,8)	24	<1	<1	<1	<1	<1	<1
3a (III:6,7,8)	107	<1	<1	<1	<1	<1	<1
3b (III:6,3,4)	92	<1	<1	6	10	430	80
4a (IV:3,4)	567	40	<1	<1	30	500	9
4b (IV:6)	71	<1	<1	10	100	<1	8
5a (V:3,4)	161	<1	<1	<1	3	<1	<1
5b (V:7,8)	118	<1	<1	<1	<1	<1	<1
X (−:7,8)	387	<1	<1	<1	<1	<1	<1
Y (−:3,4)	98	5	5	5	3	240	10
6 (VI:−)	65	<1	<1	<1	<1	<1	<1
4bR[b] (−)	<1	<1	<1	<1	<1	<1	<1

[a] ELISA endpoint titers were calculated as the reciprocal serum dilution at 405 nm × 100 min giving the absorbance value 0.1.
[b] The 4bR LPS carries an *Escherichia coli* R3 core completely devoid of O antigen.

3,4. MASF Y-2 antibodies bound exclusively to Y LPS. MASF Y-3 antibodies bound to LPSs of serotypes 2a, 3b, 4b, and Y. MASF Y-4 antibodies bound to 2a, 3b, 4a, 4b, 5a, and Y LPSs. The rat monoclonal antibody MASF Y-5 bound to LPSs of serotypes 2a, 3b, 4a, and Y. Thus several binding patterns were seen with the MASF Y-1 to Y-5 monoclonal antibodies. For comparison, a conventional rabbit Y antiserum was tested and shown to bind to LPSs of serotypes 2a, 3b, 4a, 4b, and Y (Table 1). MASF Y-5 was the monoclonal antibody that most resembled the classical rabbit group antigen 3,4 specificity, and hence MASF Y-5 was used for further agglutination studies.

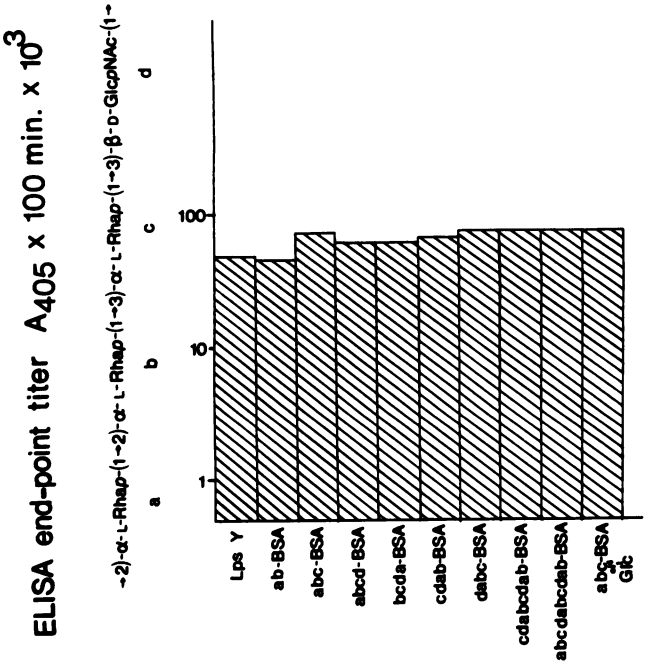

FIG. 4. ELISA endpoint titration of ascitic fluid from a hybridoma recognizing group antigen 1 in *S. flexneri*. MASF B (IgM). Designations of the various saccharides follows the nomenclature in reference 2.

Specificity test of *S. flexneri* monoclonal antibodies in direct agglutination and coagglutination using sensitized *S. aureus* Cowan 1 bacteria. To investigate whether the specificities seen in ELISA for the monoclonal antibodies described herein were of general validity, they were used with sensitized *S. aureus* Cowan 1 bacteria in coagglutination (or direct agglutination if IgM) of a large number of *S. flexneri* strains (Table 2).

MASF IV-1 and MASF IV-2 antibodies. MASF IV-1 antibodies recognized all but one of the *S. flexneri* 4a strains (O antigens IV:3,4) and all serotype 4 strains (O antigen IV only; IV:−), but neither of the two 4b (O antigens IV:6) strains available. The MASF IV-2 antibodies agglutinated the two 4b strains, 14 of 20 4a strains (among them the strain that was negative for MASF IV-1), and 19 of 21 serotype 4 strains. These results were consistent even when single colonies from different 4a strains, positive for MASF IV-1 and negative for MASF IV-2, were investigated. Thus the differences in specificity seen in ELISA titrations (Fig. 2a and b, Table 2) were confirmed: neither of the two antigen IV monoclonal antibodies agglutinated all *S. flexneri* 4a and 4b strains, but used together they identified all strains as belonging to serotype 4. The MASF IV-1 antibodies also agglutinated all strains of the provisional serotype E1037 (4X); the MASF IV-2 antibodies were unreactive with these strains.

MASF V-1 antibodies. The coagglutination with the MASF V-1 antibodies gave clear-cut results. All *S. flexneri* strains of serotype 5, whether 5a or 5b, were agglutinated. None of the other *S. flexneri* strains scored as positive (Table 2).

MASF VI-1 antibodies. Similarly, the MASF VI-1 antibodies, specific for *S. flexneri* serotype 6, coagglutinated all *S. flexneri* type 6 strains, with no false-positives among the other *S. flexneri* strains tested (Table 2).

MASF B and MASF Y-5 antibodies. The binding pattern of the MASF B antibodies obtained from the *S. flexneri* Y-immunized animals indicated that the antibody might be specific for an antigenic determinant common to all *S. flexneri*. This proved to be true when tested in direct agglutination (Table 2). All *S. flexneri* strains were positive, including *S. flexneri* serotype 6 strains. Surprisingly, when *S. dysenteriae* serotype 1 strains were tested in agglutination with MASF B, 19 of 19 tested strains were positive. Single strains of *S. dysenteriae* serotypes 2 through 9, as well as *S.*

TABLE 2. Agglutination and coagglutination using *S. aureus* Cowan 1 sensitized with monoclonal antibodies specific for *S. flexneri* types IV, V, and VI, group antigen 3,4, and all *S. flexneri*

S. flexneri serotype (antigenic formula)	No. of strains positive/no. of strains tested					
	MASF IV-1 IgG3	MASF IV-2 IgG2c	MASF V-1 IgG3	MASF VI-1 IgG3	MASF Y-5 IgM	MASF B IgM
1a (I:4)	0/15	0/15	0/15	0/15	15/15	15/15
1 (I:−)[a]	0/11	0/11	0/11	0/11	0/11	11/11
1b (I:6)	0/29	0/29	0/29	0/29	0/29	29/29
2a (II:3,4)	0/64	0/64	0/64	0/64	64/64	64/64
2b (II:7,8)	0/23	0/23	0/23	0/23	0/23	23/23
3a (III:6,7,8)	0/25	0/25	0/25	0/25	0/25	25/25
3b (III:6,3,4)	0/17	0/17	0/17	0/17	17/17	17/17
4a (IV:3,4)	19/20	14/20	0/20	0/20	20/20	20/20
4 (IV:−)[a]	21/21	19/21	0/21	0/21	0/21	21/21
4b (IV:6)	0/2	2/2	0/2	0/2	0/2	2/2
4X (IV:7,8)[b]	43/43	0/43	0/43	0/43	0/43	43/43
5a (V:3,4)	0/18	0/18	18/18	0/18	18/18	18/18
5b (V:7,8)	0/5	0/5	5/5	0/5	0/5	5/5
X (−:7,8)	0/9	0/9	0/9	0/9	0/9	9/9
Y (−:3,4)	0/20	0/20	0/20	0/20	20/20	20/20
6 (VI:−)	0/24	0/24	0/24	24/24	0/24	24/24

[a] Strains of serotype I:− and 4:− were negative for group antigen 6 and group antigen 3,4 as judged by coagglutination with MASF 6-2 (10) and Wellcome rabbit anti-*S. flexneri* Y antiserum (ZF 10, lot K 025910), respectively.
[b] 4X serotype refers to the provisional serotype E1037 described by Bernhard Rowe (personal communication).

boydii strains of serotypes 1 through 15, were all negative in agglutination with MASF B.

The MASF Y-5 (IgM) antibodies were tested in direct agglutination against the *S. flexneri* strains. All strains of serotypes 1a, 2a, 3b, 4a, 5a, and Y were agglutinated, whereas all serotype 4 strains (IV:−) were negative. All type 1 strains (I:−) were also unreactive. None of the 1b, 2b, 3a, 4b, 5b, X, or serotype 6 strains was agglutinated (Table 2).

ELISA antigen saturation experiments with different 4a, 4b, and 4X LPSs with MASF IV-1 and IV-2. To investigate more closely the binding specificities of the MASF IV-1 and IV-2 antibodies, antigen saturation experiments in ELISA were performed with native and de-O-acetylated LPS. De-O-acetylation removes alkali-labile groups such as *O*-acetyls from LPS. This will transform a serotype 4b LPS into a 4a, since the only structural difference in between serotype 4a and 4b LPSs is the *O*-acetyl on C2 of rhamnose III (30) (Fig. 1a). As antigens, three different 4a LPSs (labeled no. 1, 2, and 3), one 4b LPS, and one 4X LPS were used (Table 3). In these tests the MASF IV-1 antibodies bound to 4a LPS no. 2 and 3 and to the 4X LPS, as well as to the de-O-acetylated counterparts of these LPSs, but were negative for the 4b LPS and the no. 1 4a LPS irrespective of whether it was de-O-acetylated or not. The IV-2 antibodies bound both to the native and de-O-acetylated forms of 4a LPSs no. 1 and 2 and to 4b LPS, but not to the no. 3 4a LPS or to 4X LPS. Both monoclonal antibodies were positive for 4a LPS no. 2. The no. 1 and 2 serotype 4a LPSs, as well as the 4b and 4X LPSs, have been subjected to proton nuclear magnetic resonance. The 4a LPSs were shown to contain the signals expected for a 4a LPS. Likewise, the 4b LPS exhibited all signals expected for a 4b LPS. In the 4X LPS we could not, in preliminary experiments, detect any signal from α-D-Glc*p*-(1→6)-β-D-Glc*p*NAc, i.e., the antigenic determinant for the type antigen IV in *S. flexneri*. These investigations are presently in progress (data not shown).

TABLE 3. Antigen saturation ELISA with *S. flexneri* LPS of serotypes 4a, 4b, and provisional serotype E1037 (4X)

LPS serotype	Antigen needed (μg) for an $A_{405} \times 100$ min of 0.1	
	MASF IV-1	MASF IV-2
4b	>50	0.9
De-O-4b[a]	>50	0.6
4X[b]	0.9	>50
De-O-4X	1.0	>50
4a no. 1	>50	1,8
De-O-4a no. 1	>50	0.1
4a no. 2	0.5	0.6
De-O-4a no. 2	1.0	0.06
4a no. 3	0.8	>50
De-O-4a no. 3	0.7	>50

[a] De-O-acetylated LPS; see Materials and Methods.
[b] Refers to the type strain E1037 of the provisional serotype 4X.

DISCUSSION

The serology of *S. flexneri* has been enigmatic for most microbiologists, particularly the relationships between the different epitopes in the O-antigenic polysaccharide chain of the LPS. The structural elucidation of the polysaccharides of the LPS by B. Lindberg and co-workers in the 1970s (29–31) laid a foundation for an understanding of the immunochemical specificities. Such an understanding is of interest not only to the specialists in the field. In studies of the protective immunity elicited by immunization of humans and monkeys the protection has been described both as serotype specific, i.e., O-antigen specific (21, 22, 38, 39), and as species specific, i.e., protective against all serotypes of *S. flexneri* (26, 37). For the elucidation of such a crucial question it is evident that it is of interest to investigate against which determinant(s) of the O-polysaccharide chain antibodies are elicited; i.e., are they formed against epitopes common to all *S. flexneri* strains, or are most antibodies formed against type-specific epitopes? One common epitope is the group 3,4 antigen. Hitherto undefined in structural terms, it is found in serotypes 1a, 2a, 3b, 4a, 5a, and Y. These serotypes are among the most commonly isolated from cases of shigellosis (40), and therefore studies of the common 3,4 epitope are of

interest. We isolated five monoclonal antibodies, MASF Y-1 to Y-5, for an initial study of their binding specificities (Table 1).

The binding specificity of the mouse monoclonal antibodies MASF Y1 through Y4 indicated that they all recognized different structural domains of the *S. flexneri* O antigen, but that none of them defined the complete group 3,4 antigen. From the structural analyses of the O antigens of *S. flexneri* it can be deduced that substitution of rhamnose I (unit *a*) with D-glucose that is linked α-1,3 completely abolishes group antigen 3,4 activity (serotypes 2b, 3a, 5b, and X). Likewise, the concomitant presence of an *O*-acetyl linked to C2 of rhamnose III (unit *c*) and a D-glucose linked α-1,4 or α-1,6 to the *N*-acetylglucosamine (unit *d*) (as in serotypes 1b and 4a) is accepted in the absence of the *O*-acetyl linked to C2 of rhamnose III (unit *c*). The group antigen 3,4 epitope could then be defined as the intrachain determinant *cda*, in which the *a* unit cannot be substituted whereas the *c* or *d* unit can, but not simultaneously (N. I. A. Carlin, D. R. Bundle, and A. A. Lindberg, J. Immunol., in press).

The binding specificity of the MASF Y-5 antibodies seems to be closest to a "classical" group antigen 3,4 specificity. In ELISA these antibodies bound to LPSs of serotypes 2a, 3b, 4a, and Y, and in agglutination also some strains of serotypes 1a and 4a (group antigen 3,4 positive as judged by the polyvalent rabbit antiserum) and all 5a strains were agglutinated (Table 2). The discrepancies in the reactivity of the MASF Y-5 antibodies seen in ELISA and agglutination were probably caused by the low affinity of these antibodies.

The equilibrium constant for the MASF Y-5 antibodies has been determined to be 5×10^4 liters/mol (Carlin et al., in press). ELISA, being an affinity-dependent assay (6), can thus give a negative result for a given antigen-antibody reaction, while the same reaction can be scored as positive in coagglutination, where the functional affinity (avidity) is much higher due to the multivalency of antibodies and antigen. We have seen this also for other monoclonal anti-carbohydrate antibodies (10).

The *S. flexneri* MASF B antibodies. The specificity of the MASF B antibodies indicated that they recognized an epitope common to all *S. flexneri* (including serotype 6) and *S. dysenteriae* type 1 strains. Early work by Boyd (4) and Wheeler (48) suggested the presence of a common group antigen labeled as 1 in all *S. flexneri* strains. This hypothesis was challenged by other (46; S. Madsen, Ph.D. thesis, University of Copenhagen, Copenhagen, Denmark, 1949) who were unable to find this common antigenic determinant. Structural studies of *S. flexneri* and *S. dysenteriae* type 1 revealed that all have L-rhamnopyranosyl residues linked α-1,2 or α-1,3 in their repeating units (Fig. 1) (16, 17, 30). In a glycoconjugate ELISA, the MASF B antibodies bound all glycoconjugates tested (Fig. 4), including the α-L-Rha*p*-1→2-α-L-Rha*p*-BSA conjugate. This shows that the MASF-B antibodies recognize an epitope as small as a disaccharide. We propose that, based on our findings, the group antigen 1 epitope can be considered as revived. The importance of immunity against this structural entity, common to all *S. flexneri*, in protective immunity remains to be established. This can be accomplished using an appropriate animal model for shigellosis.

Type antigen V- and VI-specific monoclonal antibodies. The *S. flexneri* type antigens V and VI monoclonal antibodies (MASF V-1 and VI-1) gave unambiguous results in both ELISA and coagglutination (Fig. 3, Table 2). The MASF V-1 antibodies bound to both 5a and 5b LPSs in ELISA and correctly identified all *S. flexneri* 5a and 5b bacteria in coagglutination. This implies that the antigenic determinant for these antibodies is the D-glucose that is linked α-1,3 to rhamnose II of the O chain of *S. flexneri* (Fig. 1). The MASF VI-1 antibodies bound only to *S. flexneri* type 6 LPS in ELISA. In coagglutination, all 24 strains of *S. flexneri* type 6 bacteria were scored as positive. The excellent specificities of the MASF V-1 and VI-1 antibodies are in agreement with our previous results obtained with *S. flexneri* O-antigen-specific monoclonal antibodies (8–10).

The *S. flexneri* type antigen IV epitope. The *S. flexneri* type antigen IV is found only in strains of serotypes 4a and 4b. Structural studies of the *S. flexneri* O antigens have shown that the common structure is the α-D-Glc*p*-1→6-β-D-Glc*p*N Ac group (30) (Fig. 1). The 4a bacteria also carry specificity for the group antigen 3,4, and 4b bacteria carry group antigen 6 specificity. In recent years a new provisional serotype, E1037 (4X), has been found (B. Rowe, personal communication). Based on agglutination studies with absorbed rabbit antisera, type antigen IV and group antigen 7,8 specificities were found in serotype 4X bacteria. We failed in our attempts to produce a type antigen IV-specific monoclonal antibody recognizing the type IV antigen in 4a, 4b, and 4X strains. In 25 fusions with mouse spleen cells primed with 4a or 4b bacteria, involving the testing of more than 1,200 positive culture supernatants in ELISA, no antibody could be found that recognized all three antigens. From fusions with spleen cells primed with 4a bacteria, only hybridomas producing antibodies with specificity for 4a and 4X LPS could be obtained (MASF IV-1). Fusions with spleen cells primed with 4b bacteria resulted only in the recovery of group antigen 6-specific antibodies. These results suggested that at least the mouse strains used might be genetically restricted in their recognition of the type antigen IV (1).

To circumvent these problems we immunized LOU/C rats with two different *S. flexneri* 4a strains; one of them was the 4a strain negative for MASF IV-1 in coagglutination (Table 2). This LPS was also used for the preparation of LPS 4a no. 1 (Table 3). When the strain was used as an immunogen in BALB/c or C57BL/6 mice, no positive hybridomas could be isolated after three different fusions. Surprisingly, hybridomas secreting antibodies specific for both 4a and 4b LPS, but not for 4X LPS, could readily be isolated from both fusions by using rat spleen cells (MASF IV-2; Fig. 2b).

When the specificity of the MASF IV-1 and IV-2 antibodies was tested by coagglutination, the MASF IV-1 antibodies agglutinated all 4X and 4− strains, all but one of the 4a strains, but none of the 4b strains. With the exception of the negative 4a strain, this was in accord with the ELISA results. The MASF IV-2 antibodies agglutinated 14 of 20 4a strains and 19 of 21 4− strains, both 4b strains, but none of the 4X strains. These somewhat conflicting results led us to investigate more closely the binding specificity of the MASF IV-1 and IV-2 antibodies in ELISA antigen saturation experiments (Table 3). The fact that not all *S. flexneri* serotype 4a strains reacted identically with the monoclonal antibodies made us select three isolates and prepare their LPSs for further studies. These were designated 4a no. 1 (MASF IV-2 reactive), 4a no. 2 (MASF IV-1 and IV-2 reactive), and 4a no. 3 (MASF IV-1 reactive). One 4b and one 4X LPS were also used, as well as alkali-treated preparations of all five LPSs. In these ELISA experiments, the MASF IV-1 antibodies were negative for the native as well as for the de-O-acetylated 4b LPSs (which will functionally be a 4a LPS), proving that the specificity of these antibodies was not directed against the type IV epitope (Table 3). Furthermore, MASF IV-1 bound strongly to both the native and de-*O*-

acetylated forms of the 4X LPS, indicating that it recognized an alkali-stable antigenic determinant on this LPS. MASF IV-1 also bound strongly to two of the three 4a LPSs (no. 2 and 3). One of these (no. 2) has been shown to be a true 4a LPS by proton nuclear magnetic resonance, but that is also true for the 4a LPS no. 1, for which MASF IV-1 has no binding activity. These data strongly indicate that in most but not all 4a strains there is an unknown alkali-stable antigenic determinant present in minute amounts. This antigenic determinant (4X) is present also on Y strains (leading to the erroneous classification of these strains as 4a) and on some subserotype X strains, i.e., 4X strains. We are currently doing structural analytical work on such LPSs.

The MASF IV-2 antibody bound to the 4b LPS and the two 4a LPSs with confirmed structure, indicating that it recognizes the α-D-Glc*p*-1→6-β-D-Glc*p*NAc determinant in the serotypes 4a and 4b. The presence of an *O*-acetyl on C2 of rhamnose III does not markedly affect binding.

Variability in *S. flexneri* O antigens. A factor underlying the problems of accurately defining the specificity of all the monoclonal antibodies may be the variability of the *S. flexneri* O polysaccharide chain. As indicated by Edwards and Ewing, the antigenic formulae for the different *S. flexneri* strains must be considered as abbreviations of a more complex reality (18). What is designated as group antigen 3,4 specificity in one serotype is not necessarily the same in another serotype. This follows from the different positions of the substituents added to the repeating unit (Fig. 1). The binding specificity of several *S. flexneri* Y monoclonal antibodies, with potential group antigen 3,4 specificity, showed that they vary in their ability to recognize the various 3,4-group antigen-containing serotypes (Table 1). Furthermore, it is likely that at least some of the substituents are subject to form variation; i.e., the antigen is either expressed or not. Such variations were reported early by Boyd (3) and Takita (44). The inherent antigenic variability makes the production of specific absorbed polyclonal antisera next to impossible, and hence the preparation of immunochemically well-characterized monoclonal antibodies is an important step. Furthermore, these antibodies may prove to be valuable reagents in the studies of protective immunity against *Shigella* bacteria.

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