Salmonella Serogroups C2 and C3 Identified by Agglutination Using an Immunoglobulin G3(κ) Monoclonal Antibody (32-1-E3) Reactive with a Somatic Factor 8-Like Polysaccharide Antigen

PAUL S. DUFFEY,* JUNE C. KANI, JADE O. LEE, WARREN J. HILL, AND ROBERT KOKKA†

Microbial Diseases Laboratory, California Department of Health Services, 2151 Berkeley Way, Berkeley, California 94704

Received 8 June 1992/Accepted 1 September 1992

An immunoglobulin G3(κ) monoclonal antibody (MAb), MAb 32-1-E3, which was prepared in BALB/c mice by using a heated, alcohol-acetone-extracted Salmonella newport CDC 50 antigen, reacted with protein-free lipopolysaccharides from Salmonella groups C2 (O:6,8) and C3 (O:8) but not with those from any other serogroup tested. Sodium periodate did not inhibit antigen reactivity, which was consistent with its identity as the abequrose-containing disaccharide O:8 antigen. Reactivity was inhibited by competition with serogroup C2 (O:6,8) and C3 (O:8) antigens but not with non-O:8 antigens. Reactivity was also inhibited by preincubation of the antigen with polyclonal rabbit antisera group C2 or C3 antibodies but not with antisera to serogroup C1 or other Salmonella serogroups. The MAb agglutinated with all strains of Salmonella serogroups C2 and C3 tested but not with other bacteria. Agglutination was inhibited by preabsorbing the MAb with either of two serogroup C3 Salmonella strains, S. virgillia CDC 189 or S. haardi MDL 83A4545, which contain only O:8, but not by preabsorption with O:8-negative S. cholerasuis MDL 81A7623 (group C1; O:6,7), S. paratyphi type B CDC 157 (group B; O:1,4,5,12), or Escherichia coli (O:157) (which contains no Salmonella serogroup antigens). The MAb reacted strongly (++ agglutination) with all 140 wild-type strains of group C2 and C3 Salmonella spp. tested and showed no reaction with any of 1,324 wild-type strains of non-C2 or non-C3 Salmonella spp. tested. The MAb is useful as a replacement for absorbed, polyclonal, single-factor O:8 antiserum to discriminate Salmonella serogroups C2 and C3 from serogroup C1.

Salmonella serogroups C1, C2, and C3 differ in the presence of factors O:6, O:7, O:8, O:14, and O:20 (5, 13). Antigen factor O:6 occurs in two variants (4). In addition to factors O:6 and O:7, factor O:14 is found only on some strains of group C1 bacteria that contain a lysogenic phage, and in addition to factor O:8, factor O:20 is found only on lysogenic strains of C3 bacteria (5, 18). Identification of strains in these serogroups requires so-called single-factor antisera that are prepared by absorbing antisera group C1, C2, and C3 sera to remove reactivity to all but the antigen of interest, i.e., to produce single-factor O:6, O:7, O:8, O:14, or O:20 antiserum (5, 13). Absorbed, single-factor polyclonal antisera are routinely produced and used in our laboratory to separate subgroups C1 (factors O:6 and O:7) from subgroups C2 (factors O:6 and O:8) and C3 (factor O:8). However, single-factor antisera are made only in small volumes, have low usable titers (i.e., often must be used undiluted or diluted 1:2), often must later be reabsorbed to remove low levels of cross-reactivity that remain after initial absorption, and may produce less than optimum agglutination with bacteria containing the homologous somatic antigen factor while retaining a detectable level of reactivity against extraneous antigens.

Monoclonal antibody (MAb) reagents that are reactive in slide agglutination tests for Salmonella serogroup identification (1, 4, 15–18, 26–28) have been described. Once they are prepared and characterized, cultures of MAb-secreting cells can be used to regenerate exquisitely specific antibody reagents as needed, without absorption and by simplified standardization procedures, since their specificity does not change (11). Thus, it should be possible, over time, to prepare sets of MAb reagents suitable for identifying serogroups of Salmonella.

We report here the characteristics of an immunoglobulin G3(κ) [IgG3(κ)] MAb reagent that detects a factor O:8-like antigen located on Salmonella serogroup C2 and C3 lipopolysaccharide (LPS) moieties. The MAb is useful as a replacement for the polyclonal, absorbed single-factor O:8 antiserum commonly used for Salmonella serogroup determination.

MATERIALS AND METHODS

Salmonella strains. Those strains recommended for preparation and characterization of serogrouping reagents (4, 13) were obtained originally from the Enteric Diseases Laboratory, Centers for Disease Control, Atlanta, Ga. (CDC strains), or were clinical isolates submitted to the Microbial Diseases Laboratory, California Department of Health Services, for identification (MDL strains). The strains were found to be suitable for use as immunizing antigens or for evaluating antiserum. Stock strains were maintained at −70°C in a mixture of sheep blood and alundum. Working cultures were maintained at 4°C on heart infusion agar slants (Difco, Detroit, Mich.). For isolation, the bacteria were streaked onto heart infusion agar plates, and single colonies were obtained for use. The identities and antigenic contents of all strains were confirmed before use by using standard identification methods and antisera (5). The antisera were prepared by the Biologics Unit, Microbial Diseases Laboratory,
California Department of Health Services, by standard procedures (5, 13) and are the reagents used routinely for identification of *Salmonella* spp. in our laboratory.

**Mice.** Female BALB/c mice (age, 6 weeks) were from colonies maintained by the Division of Laboratories, California Department of Health Services. They were housed in a National Institutes of Health- and American Association for the Accreditation of Laboratory Animal Care-certified facility and were maintained on commercially supplied mouse chow and water ad libitum.

**Hybridomas.** Mice (age, 8 to 10 weeks) were injected intraperitoneally weekly for 4 weeks with 0.2 ml of a heat-killed, alcohol-acetone-extracted *S. newport* CDC 50 (serogroup C2; O:6,8) antigen (22) suspended at ca. 0.5 mg/ml in 0.5% phenolized (pH 7.2), 0.15 M phosphate-buffered physiological saline (PBS). Two days following a final 0.1 ml intravenous dose, mouse spleen cells (10<sup>6</sup>) in Hanks’ balanced salt solution (catalog no. 310-4060AJ; Gibco, Gaithersburg, Md.) were fused with 10<sup>7</sup> log-phase P3x63-ag8.653 plasmacytoma cells (CRL 1581; American Type Culture Collection, Rockville, Md.) using 50% polyethylene glycol 4000 (catalog no. P-146; Fischer Scientific, Fairlawn, N.J.) and were cultured essentially as described by Kennett et al. (11).

**Thymocyte-conditioned medium.** Thymocytes from 4-week-old mice (8) were adjusted to a concentration of 2 x 10<sup>7</sup>/ml in Dulbecco modified Eagle medium (catalog no. 340-1600; Gibco) with 20% supplemented, defined calf serum (catalog no. A2151-2; Hyclone, Logan, Utah), incubated overnight in a T-75 flask as described above, and then stored frozen at −70°C. When needed, the medium was thawed at room temperature and mixed 1:1 with fresh Dulbecco modified Eagle medium.

**Selection and cloning of MAb-producing cell lines.** Hybridomas were subcultured onto 24-well cluster plates (catalog no. 3524; Costar, Cambridge, Mass.), and the supernatants were tested for antibody against *S. newport* CDC 50 by an enzyme immunosorbent assay (EIA) as described below. Positive cultures were serially diluted and cloned twice in thymocyte-conditioned Dulbecco medium containing 0.24% agarose (lots 12952 or 11939;Sigma, St. Louis, Mo.; FMC Bioproducts, Rockland, Maine) (11). EIA-positive cultures were grown to a cell density of 5 x 10<sup>5</sup> cells per ml in flasks (T-25 and T-75 flasks; catalog nos. 25100 and 25110; Costar), and aliquots (1.0 ml, 5 x 10<sup>5</sup> hybridoma cells) in calf serum with 5% dimethyl sulfoxide (catalog no. D-128; Fisher Scientific Co., Pittsburgh, Pa.) were frozen to −96°C at a rate of −1°C/min by using a model CRC-1 temperature rate controller and CRFC-1 freezing chamber (Linde Division, Union Carbide, New York, N.Y.).

**Immunoglobulin class and isotype.** The immunoglobulin heavy chain class and isotype and light chain class were determined by an EIA antibody-capture method (Immunos-elect kit; catalog no. 96600SA; Gibco-Bethesda Research Laboratories) that was performed in Immulon II 96-well plates according to the manufacturer’s instructions.

**MAb-containing mouse ascites.** Cloned hybridoma cells (ca. 2.5 x 10<sup>6</sup>) were injected intraperitoneally into 12-week-old BALB/c mice that were treated intraperitoneally 2 weeks previously with 1 ml of Pristane (catalog no. T2,280-2; Alltech Associates, Milwaukee, Wis.) (6). Seven to 10 days later, 5 to 10 ml of pooled ascites from each mouse was harvested, allowed to clot at 37°C, and then clarified by centrifugation at 15,000 rpm (J2-21M centrifuge and JA-20 rotor; Beckman Instruments, Fullerton, Calif.) for 60 min. Sterile ascites containing no MAb were prepared by injecting the mice intraperitoneally with 0.25 ml of Freund’s complete adjuvant (Difco) (20) and were used as a negative control for some experiments. After centrifugation, the ascites supernatant was filtered through a 0.2-μm-pore-size membrane filter (catalog no. 62440; Schleicher & Schuell, Keene, N.H.) and stored at 4°C.

**Absorption.** A single Roux bottle (Corning 1290; VWR Scientific, San Francisco, Calif.) containing 180 ml of brain heart infusion agar (Difco) was inoculated with 1.5 ml of an 18- to 24-h broth culture of bacteria grown at 35°C. The growth was distributed with a small, sterile glass rod and was then incubated overnight at 35°C. The growth was suspended in 10 ml of 0.5% phenolized PBS, diluted into 2-ml portions, and centrifuged for 30 min at 10,000 rpm (J2-21M centrifuge and JA-20 rotor; Beckman). The packed cells were resuspended in 2 ml of ascites, incubated for 2 h at 50°C and then at 4°C overnight, and centrifuged at 10,000 rpm as described above; the absorbed antibody was then harvested. The procedure was repeated once.

**Protein- and nucleic acid-free LPS.** Overnight cultures in Roux bottles were extracted by the previously described hot phenol-water method (7, 29), and the extract was then treated as follows to remove nucleic acids and residual proteins. The LPS, which was suspended in 120 ml of class I reagent-grade deionized water (LPS-free), was mixed with 10-mg amounts of RNase A (catalog no. R-9005; Sigma and Difco; type II; catalog no. D-4527; Sigma) and incubated for 1 h at room temperature (ca. 23°C). Ten milligrams of protease K (type XXVIII; catalog no. P-4914; Sigma) was then added, and the mixture was incubated at 60°C for 1 h. After centrifugation at 9,800 x g for 30 min at 5°C (Beckman J21C centrifuge), the supernatant was recentrifuged at 90,000 x g for 4 h at 5°C (Beckman L-2 ultracentrifuge with a 25.2 rotor); and the pellet was resuspended in 120 ml of class I reagent-grade water and recentrifuged twice more, resuspended in 6 ml of class I reagent-grade water, divided into 2-ml aliquots, and freeze-dried. The yield was approximately 175 mg of total material.

**Antibody detection.** An EIA (Vectastain ABC kit; catalog no. PK-4000; Vector Laboratories, Burlingame, Calif.) was performed as follows. Labeled antigen (1 μg) (P3x63-ag8.653 supernatants) in 120 μl of 10% ascites (Difco) and 0.2% Pristane (Sigma) was incubated for 1 h in 12-well cluster plates. The plates were washed five times with PBS containing 0.05% (vol/vol) EIA-grade Tween 20 (catalog no. 170-6531; Bio-Rad). Then, 100 μl of undiluted hybridoma supernatant or mouse serum or ascites was added to 200 μl of 0.06 M carbonate-bicarbonate buffer (pH 9.5), was dispensed at 200 μl per well into 96-well Immulon II microtiter plates (Dynatech, Chantilly, Va.) and incubated overnight at 5°C. The plates were then countercoated with 0.01 M PBS (pH 7.2) containing 3% EIA-grade gelatin (catalog no. 170-6537; Bio-Rad Laboratories, Richmond, Calif.) for 5 min at room temperature. *S. newport* CDC 50 antigen powder (22) suspended in PBS at 0.05 mg/ml was added (100 μl per well), adsorbed for 1 h at room temperature, and washed five times with PBS containing 0.05% (vol/vol) EIA-grade Tween 20 (catalog no. 170-6531; Bio-Rad). Then, 100 μl of undiluted hybridoma supernatant or mouse serum or ascites was added to 200 μl of 0.06 M carbonate-bicarbonate buffer (pH 9.5) containing 1% (wt/vol) gelatin (EIA grade; Bio-Rad) and 1% (vol/vol) normal rabbit serum, was pipetted into pairs of wells and incubated for 1 h at 35°C. The supernatant from the P3x63-ag8.653 myeloma culture, which does not secrete antibody, or sterile ascites was used as a negative control. Mouse antiserum to the *S. newport* CDC 50 antigen, diluted 1:5,000, was used as a positive control. After incubation, the plates were washed five times as described above and were developed according to the manufacturer’s instructions (Vector) by using ABTS staining reagent (catalog no. 506200; Kirkegaard and Perry, Gaithersburg, Md.). The plates were incubated for 1 h at room temperature in the dark and then read.
on a model EL309 dual-wavelength EIA plate reader (Biotek Laboratories, Winooski, Vt.) at 405 and 540 nm. The readings obtained at 540 nm were subtracted from those obtained at 405 nm. Wells that gave net readings of ≥0.5 absorbance unit (AU) on initial screening or ≥0.8 AU after cloning, following subtraction of the AU of the negative control, were considered positive.

Agglutination was done by a standard slide assay with twofold dilutions, in 0.85% NaCl (saline), or serum or ascites against 50% ethanol-saline suspensions of Salmonella cultures grown overnight in brain heart infusion agar slants (5).

Polyacrylamide gel electrophoresis. Proteinase K-treated LPS, which was prepared by the procedure of Hitchcock and Brown (9), as modified by Kokka et al. (12), was electrophoresed as described by Stein et al. (24), except that a 6.5% stacking gel was used and 0.02% bromophenol blue (catalog no. 161-0404; Bio-Rad) was included in the sample buffer as an indicator dye. The buffer system MZE 3328.IV devised by Jovin (10) was used as described by Moos et al. (19). Electrophoresis was performed at a constant 8.0 mA per gel for approximately 1.5 h at room temperature by using a model PS 500X regulated power supply (Hoeffer Scientific, San Francisco, Calif.) until the indicator dye was approximately 5 mm from the bottom of the gel. The gels were then either stained directly (2, 9, 12, 25) by using a silver staining kit (catalog no. 161-0443; Bio-Rad) and photographed by using electrophoresis duplicating paper EDP (Kodak, catalog no. 182-7831; Kodak) or used for immunoblot assays as described below.

Immunoblot assays. LPSs from sodium dodecyl sulfate (SDS)-polyacrylamide gels were electroblotted (21) onto polyvinylidene membranes (pore size, 0.45 μm; Immobilon-P; catalog no. IPVH 151 50; Millipore, Milford, Mass.) essentially as described by Stein et al. (24) by using the carbonate buffer (pH 9.9) system of Dunn (3) and by adding 0.05% SDS. Transfers were performed with a Mini Trans-Blot electrotransfer transfer cell (catalog no. 170-3930; Bio-Rad) and a Hoeffer PS500X power supply at a constant 400 mA for 1 h at <20°C. EIA staining (23) was as follows. The membranes were wetted in absolute methanol for 3 s and then rinsed in reagent-grade, deionized water and blocked in PBS containing 3% gelatin for 30 min with gentle agitation. After aspirating the PBS, MAb ascites diluted 1:640 or 50% glycineated rabbit anti-Salmonella serogroup A serum diluted 1:40 in PBS containing 1% gelatin (sufficient to cover the membranes) was added, and the membranes were gently agitated for 1 h and washed three times in PBS–1% gelatin. A protein A-horseradish peroxidase conjugate (catalog no. 170-6522; Bio-Rad) diluted 1:1,000 in PBS containing 1% gelatin was then added (sufficient to cover the membranes), and the solution was gently agitated for 30 min. The membranes were again washed three times, 4-chloro-1-naphthol (horseradish peroxidase color development reagent; catalog no. 170-6537; Bio-Rad) was added (sufficient to cover the membranes), and the solution was incubated at room temperature with gentle agitation until the color development was judged to be sufficiently intense (about 10 min).

The solution was then rinsed twice in deionized water, air dried, and stored in the dark (22, 25, 28).

Periodate oxidation. The periodate oxidation method of Woodward et al. (30) was used. Individual Immobilon-P membrane strips containing LPS antigens were immersed in methanol for 3 s, rinsed in reagent-grade deionized water for 1 to 2 min, and then washed once for 5 min in 10 ml of 50 mM sodium acetate buffer (pH 4.5). Sodium periodate (catalog no. S-1147; Sigma) in 50 mM sodium acetate buffer (pH 4.5; 10 ml) was freshly prepared at 10, 50, and 200 μM. Individual strips were immersed in the dark for 1 h, gently agitated with a laboratory rotator, and then washed three times for 10 min each time in 10 ml of 50 mM sodium acetate buffer (pH 4.5). Freshly prepared 50 μM sodium borohydride (catalog no. S-9125; Sigma) in PBS was then added (10 ml), and the solution was gently agitated for 30 min and then washed three times for 10 min each time with gentle agitation in PBS containing 1% gelatin. The strips were then stained by the EIA method described above for the immunoblot assays, except that a protein A-alkaline phosphatase conjugate (catalog no. P-9650; Sigma) was used with a 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt)–Nitro Blue Tetrazolium substrate (catalog no. 170-641; Bio-Rad) for visualization. The monoclonal ascites was diluted 1:320.

RESULTS
Selection of reactive MAb s. Following hybridization, 199 of 824 cultures yielded hybrid colonies for a fusion efficiency of 24.2%. Of these, seven were initially reactive by EIA (≥0.5 AU) against Salmonella serogroup C2 antigens. Ascites of the three positive lines that gave the highest EIA reactions were tested by agglutination using the homologous S. newport CDC culture and strains from Salmonella serogroups A, B, C1, C2, C3, D1, D2, E1-E4, F, G1, G2, H, I, and 18 through 67. The hybrid cell line clone 32-1-E3, ascites from which was found to react equally well (agglutination titers of 1:32-1:64) with the homologous strain of S. newport and with the representative C2 and C3 strains tested and which did not react with any non-C2 or non-C3 strains tested, was cloned twice. The MAb was then reacted against protein- and nucleic acid-free LPS antigens prepared from S. montevideo MDL 1084 (group C1), S. newport CDC 50 (group C2), S. typhimurium (group B; catalog no. L-6511; Sigma), S. abortus-equus (group B; catalog no. L-5886; Sigma), S. typhi (group D; catalog no. L-6386; Sigma), and an Ra mutant (TV119) of S. typhimurium (core; catalog no. L-6016; Sigma) to determine that the MAb reacted with LPS antigens of group C2 but not with group B, C1, D, or LPS core determinants (data not shown).

Peptide composition. Supernatants from the cloned cell line were reacted with rat anti-mouse immunoglobulin iso-type MAb s in parallel with positive controls. Positive controls consisted of culture supernatants containing myeloma proteins representative of each mouse immunoglobulin heavy- and light-chain isotype, not including IgE or IgD, in an antibody-capture assay. MAb 32-1-E3 was reactive only with anti-IgG3 heavy-chain and anti-i light-chain antibodies.

Reactivity by EIA: effect of preabsorption. To further assess specificity, individual samples of ascites from the cloned hybridoma cell line were absorbed twice, as described above, with S. paratyphi B CDC 157 (serogroup B; O:1,4,5,12), S. choleraesuis MDL 81A-7623 (group C1; O:6,7), S. newport CDC 50 (serogroup C2; O:6,8), S. virgina CDC 189 or S. haardt MDL 83A-4545 (serogroup C3; O:8), or S. boecker CDC 359 (serogroup H; O:1,6,14,25). A separate sample of the 32-1-E3 ascites was carried through the procedure as if it was absorbed in order to provide a reactivity control. Serial twofold dilutions of the absorbed and control ascites were tested by EIA by using S. newport CDC 50 as the antigen. The results (Fig. 1) show that preabsorption with S. haardt or S. virgina (group C3, containing only O:8) or with S. newport (group C2, containing O:6,8) eliminated reactivity against the homologous O:8-containing antigen, while the serogroup O:8-negative
bacteria did not reduce the titer. Significantly, absorption with neither S. cholerasuis nor S. boecker, which contain serogroup factor O:6 but not serogroup factor O:8, did not reduce the titer, suggesting that the specificity of the MAb is to the serogroup factor O:8 antigen. On the basis of the titer of the unabsorbed ascites, a dilution of 1:4,000 for MAb 32-1-E3 ascites was established for further EIA studies.

**Competitive inhibition with antigens.** To further evaluate the specificity, the ascites were diluted 1:4,000 in the usual diluent that also contained serial 10-fold dilutions of *Salmonella* antigen powders in the range of 100 μg/ml to 0.01 ng/ml. These antigens included *S. agona* MDL 86A-5257 (serogroup B; O:4,12), *S. montevideo* MDL 83A-1084 (serogroup C1; O:6,7,14), *S. newport* CDC 50 (serogroup C2; O:6,8), *S. virginia* CDC 189 (serogroup C3; O:8), *S. boecker* CDC 359 (serogroup H; O:1[1],6,14[25]), and *Escherichia coli* CDC EDL 932 (enteric bacterium with no known *Salmonella* antigens). The dilutions were incubated for 1 h at room temperature, and then the EIA assay was completed in the normal fashion. Figure 2, which plots the percent inhibition of reaction versus the concentration of inhibitor, shows that the MAb reactivity was inhibited by competition with serogroup C2 and C3 *Salmonella* spp., but not by competition with serogroup C1 or other, non-serogroup C bacteria, confirming that the MAb may be reactive with somatic factor O:8.

**Competitive inhibition with antisera.** An additional assessment of the specificity in EIAs was performed as described above, except that the antigen-coated plates were preincubated for 1 h at room temperature with serial dilutions of rabbit anti-serogroup C1 (O:6,7), anti-serogroup C2 (O:6,8), anti-serogroup C3 (O:8,20), anti-serogroup B (O:4,12), and anti-serogroup H (O:6,14) in the range of 1:5 through 1:1,000 with normal rabbit serum or before incubation with MAb 32-1-E3. Figure 3, which plots inhibition versus dilution, shows that the anti-serogroup C2 and anti-serogroup C3 antisera were equally effective at inhibiting reactivity, while the anti-serogroup C1, anti-serogroup B, and anti-serogroup H antisera and normal rabbit serum were ineffective. Taken together, the results of the absorption and competition experiments argue that MAb 32-1-E3 is highly specific for a serogroup O:8-like antigen and does not show any cross-reactivity with antigens O:6, O:4, or the other serogroup antigens tested.

**Location of the factor O:8-like antigen on the LPS moiety.** To confirm the association of the O:8-like antigen with the LPS moiety, proteinase K-treated cell lysates of *S. paratyphi* A MDL 88A6213, *S. paratyphi* B CDC 157, *S. mbandaka* CDC 1002-84, *S. norwich* CDC 3209, *S. newport* CDC 50, *S. tulsan* CDC 972, *S. haardt* MDL 83A4545, *S. virginia* CDC 189, *S. kentucky* CDC 98, *S. dublin* CDC 65, *S. anatum* CDC 250-85, *S. rubislaw* CDC 185, *S. boecker* CDC 359, and *S. cerro* CDC 100 were subjected to SDS-polyacrylamide gel electrophoresis and then electroblotted onto Immobilon-P membranes. Individual membranes were incubated with MAb 32-1-E3 ascites and stained as described above. Duplicate gels were stained with a silver stain to directly demonstrate the presence of polysaccharides. Figure 4A and 4C show the results of immunological staining with MAb 32-1-E3 in Western blot (immunoblot) assays with typical LPS ladder patterns only on the O:8-containing antigens, which is consistent with the presence of the antigenic determinant on the LPS. Figure 4B and C shows duplicate silver-stained gels with typical LPS ladder patterns in all lanes.

**Periodate oxidation.** The hexose dideoxy sugar abequose, which forms the immunodominant portion of the *Salmonella* O:4 and O:8 antigens, is not destroyed by oxidation with sodium periodate (14). Thus, if the MAb indeed reacts with the O:8 antigen as an immunodominant epitope, it should not be possible to inhibit the reactivity of MAb 32-1-E3 by treatment of the antigen with even high concentrations of sodium periodate. Accordingly, proteinase K-treated *S. newport* CDC 50 and *S. paratyphi* A MDL 88A6213 immuno-blotted onto Immobilon-P membrane strips were left
untreated; were put through the series of incubations and washes used for the peridate procedure, except that no sodium periodate was used; or were treated with 10, 50, or 200 μM sodium periodate and then with 50 μM sodium borohydride. The strips containing \textit{S. paratyphi} type A antigens were stained immunologically by using rabbit anti-

serogroup A antiserum at 1:40, and the strips containing \textit{S. newport} antigens were stained with the MAb 32-1-E3 ascites diluted 1:640 as described above. Figure 5 shows that reactivity of serogroup A antiserum against the \textit{S. paratyphi} type A antigens (O:1,2,12), which are destroyed by periodate oxidation (18), was inhibited by treatment with 10 μM

![Graph 1](http://jcm.asm.org/)

**FIG. 2.** Ascites containing O:8 MAb was diluted 1:4,000, and aliquots were mixed with 10-fold dilutions (10^{-4} to 10^{-8} mg) of alcohol-acetone-extracted O antigens from \textit{S. newport} (O:6,8), \textit{S. virginia} (O:8), \textit{S. agona} (O:4,12), \textit{S. montevideo} (O:6,7), \textit{S. boecker} (O:1,6,14,[25]), or \textit{E. coli} (O:157) as competitive inhibitors against the test antigen. The mixture of antigen and diluted ascites was assayed by EIA against \textit{S. newport} (O:6,8) as the antigen, and the AU at \text{A_{405}} was recorded. The results were plotted as percent inhibition = 100 [1 – AU (test)/AU (untreated control)] against the concentration of inhibitor. The absorbance of the untreated control ascites was 2.02 ± 0.31 AU. The coefficient of variation for all points was ≤0.15. Only the antigens from \textit{S. newport} (O:6,8) and \textit{S. virginia} (O:8) produced measurable inhibition.

![Graph 2](http://jcm.asm.org/)

**FIG. 3.** Ascites containing MAb O:8 was diluted to make 1:4,000 aliquots that were mixed with 1:5, 1:10, 1:100, and 1:1,000 dilutions of rabbit antiserum (final concentrations) to \textit{S. newport} (O:6,8), \textit{S. virginia} (O:8), \textit{S. agona} (O:4,12), \textit{S. montevideo} (O:6,7), \textit{S. boecker} (O:1,6,14,[25]), and normal rabbit serum. The mixture of ascites and diluted rabbit serum was then assayed by EIA against \textit{S. newport} (O:6,8) antigen, and the AU at \text{A_{405}} was recorded. The results were plotted as percent inhibition = 100[1 – AU (test)/AU (untreated control)] against the concentration of rabbit antiserum or normal rabbit serum as inhibitor. The \text{A_{405}} of the untreated control ascites at 1:4,000 was 2.23 ± 0.17 AU. The coefficient of variation for all points was ≤0.11. Only the antisera against \textit{S. newport} (O:6,8) and \textit{S. virginia} (O:8) produced dose-dependent inhibition.
sodium periodate and that the reactivity of MAb 32-1-E3 against the S. newport antigen (O:6,8) was not inhibited by any concentration of sodium periodate tested up to 200 μM. This finding is consistent with the identity of the antigen recognized by MAb 32-1-E3 as the serogroup O:8 antigen.

Reactivity by agglutination. In order to be useful as a serogrouping reagent, it is essential that MAb 32-1-E3 be capable of agglutinating with factor O:8-containing salmonellae, viz., group C2 and group C3, but not with any other Salmonella spp. that do not contain a factor O:8 antigen. Specificity was tested by slide agglutination with serial dilutions of MAb ascites against a large panel of strains including serogroups A to I and 18 to 67. Agglutination was obtained to a titer of 1:8 (4+) against the group C2 and C3 Salmonella spp. tested, but no agglutination occurred in diluted or undiluted ascites with any other non-O:8 bacteria tested.

Comparison with group C absorbed polyclonal, single-factor antibodies. Finally, to evaluate its usefulness as a single-factor serogrouping reagent for routine use in slide agglutination assays, MAb 32-1-E3 diluted 1:4, 1:8, and 1:16 was evaluated against a panel of bacteria with absorbed polyclonal, single-factor antisera against O:6, O:7, and O:8 (used at a dilution of 1:1.5) with a panel of representative group C1 (23 strains), C2 (10 strains), and C3 (14 strains) bacteria containing these antigens. MAb 32-1-E3 reacted (4+ at 1:8, 3+ to 4+ at 1:16) with all O:8-containing group C2 and C3 Salmonella spp. tested in parallel with absorbed polyclonal anti-O:8 serum but did not react with any group C1 Salmonella spp. During 1 year of evaluation, 1,464 wild-type strains of salmonellae were examined. Of these, 140 strains were identified as group C2 or C3 Salmonella spp. by both the MAb and the absorbed polyclonal, single-factor O:8 antiserum. The 1,324 non-group C2 strains did not react with the MAb.

DISCUSSION

An MAb, 32-1-E3, described as an IgG3(κ) molecule, detected a Salmonella O:8-like antigen present on all strains of serogroup C2 and C3 bacteria tested. The cloned MAb in
ascites agglutinated with reference strains of serogroup C2 and C3 bacteria to a titer of 1:8 (4+ agglutination) but did not agglutinate with reference O:8-negative bacteria. The reaction was absorbed with S. Newport CDC 50 (group C2; O:6,8), S. Virginia CDC 189, or S. haaster MDL 83A4545 (group C3; O:9,8) but not with O:8-negative bacteria. In EIA competition experiments, MAb reactivity was competitively inhibited by O:8-containing antigens and anti-O:8 specific antibodies but not O:6- or O:4-containing antigens or anti-O:6, anti-O:4, or other non-anti-O:8 antisera. The target antigen was shown to be located on the bacterial LPS moiety and was not inhibited by treatment with sodium periodate, which is consistent with its identity as factor O:8. Thus, the MAb is an exquisitely specific and sensitive single-factor serogrouping reagent that can be used to identify O:8-containing strains of Salmonella spp. This reagent was evaluated for approximately 1 year, during which time 1464 strains of Salmonella spp. were tested. No instance occurred in which the MAb gave results different from those obtained with the single-factor, polyclonal O:8 reagent; i.e., none of the 140 strains of Salmonella serogroup C2 or C3 identified with conventional serogrouping reagents failed to react with the MAb anti-O:8 ascites, and no strain of Salmonella spp. other than group C2 or C3 reacted with the MAb. The MAb has been in routine use for over 2 years, with no instances of misidentification.

Several laboratories have reported the preparation of MAbs reactive with different Salmonella O antigens, including factors 1, 2, 4, 8, and 9; core antigen; and combinations of somatic antigens (1, 4, 16, 18, 26–28). Luk et al. (15) reported an MAb, MO8, described as an IgG3(A) immunoglobulin, that was reactive by agglutination and in ELAs with Salmonella group C2 LPS antigens but not with LPS from non-O:8-containing bacteria. However, it was found in competitive ELAs that both anti-C1 antisera (O:6,7) and anti-C2 serum (O:6,8) quantitatively inhibited the binding of MO8 to S. manhattan LPS (O:6,8), although anti-O:4 serum did not (like O:8, O:4 contains abequose as its immunodominant sugar [14]). Inhibition by anti-C1 antibody was considered to be due to steric hindrance with the O:6 antigen. In the present study, MAb 32-1-E3 was not inhibited by competition with anti-O:6 or other non-O:8 antibodies, and the titers of MAB 32-1-E3 were similar with all O:8-containing antigens, suggesting that the presence of the O:6 antigen neither enhanced nor inhibited the reactivity of MAb 32-1-E3 against factor O:8 antigen. Accordingly, the specificity of MAB 32-1-E3 is different from that of the MO8 MAB reported by Luk et al. (15), although both antibodies appear to be similarly useful in slide agglutination assays as single-factor serogrouping reagents for the detection of strains of Salmonella spp. that contain the O:8 antigen.

REFERENCES


