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Human Serum Antibody Response to the Presence of *Aeromonas* spp. in the Intestinal Tract

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A bacterial agglutination assay, a toxin-neutralizing assay, and an enzyme-linked immunosorbent assay (ELISA) were used to compare antibodies against intestinal *Aeromonas* strains in serum samples from healthy carriers (n = 6), from patients with acute (n = 15) or chronic (n = 8) gastroenteritis, from patients with gastroenteritis caused by other enteropathogenic bacteria (n = 3), and from healthy blood donors (n = 50). Evaluation of the bacterial agglutination assay showed that it was not very useful. The sensitivity of the ELISA in patients with acute or chronic aeromonas-associated diarrhea was 30% (7 of 23 patients were positive), whereas the specificity was 74% (13 of 50 healthy donors were positive). Positive results in the ELISA correlated with immunoglobulin M and immunoglobulin G responses to lipopolysaccharides of homologous *Aeromonas* strains, as determined by gel immunoradioassay and Western immunoblot analysis. The sera showed cross-reactions with heterologous *Aeromonas* strains and with *Escherichia coli* strains. The toxin-neutralizing assay was positive in 5 of 11 patients who had developed acute severe diarrhea associated with cytotoxin-producing *Aeromonas* strains (46% sensitivity), whereas only 3 of 50 healthy donors had low serum titers of cytotoxin-neutralizing antibodies (94% specificity). All five patients were over 60 years of age. Cytotoxin-neutralizing activity was not observed in the sera of other groups of patients with aeromonads in their feces. We concluded that the three different serologic assays were not consonant with one another and that only the toxin-neutralizing assay distinguished patients with acute diarrhea from other groups of patients.

The clinical significance of various *Aeromonas* species in the human intestinal tract is a controversial topic. The results of studies on the association between virulence properties of the *Aeromonas* strains and gastrointestinal infections are contradictory (1, 7, 8, 13, 14, 19, 20). We recently showed that 66 (39%) of 169 *Aeromonas* strains were isolated from patients who had a mixed infection with a known enteric pathogen, who had an underlying illness, who had previously been treated with penicillin derivates or antacids, or who had had immunotherapy (12). The age-related occurrence of various *Aeromonas* species in human feces and the occurrence of *Aeromonas* bacteremia in patients with immunosuppression also imply that host factors and previous medication are of substantial importance in the spectrum of aeromonas-associated disease (2, 10, 12–14).

By analogy with *Vibrio cholerae* infection, the human serologic response to the presence of aeromonads in the intestinal tract may be directed against exotoxins produced by *Aeromonas* spp. Some *Aeromonas* species produce a toxin that immunologically cross-reacts with cholera toxin (6, 25). On the other hand, intestinal infection by *V. cholerae* correlates better with a rise in serum antisomatic (O-antigen) antibodies than with an antitoxin response (30). This suggests that studies on the immune response to aeromonads must also give consideration to antibodies against the O antigens of aeromonads.

The purpose of this investigation was to study the human serum antibody response to fecal *Aeromonas* isolates from different hybridization groups (HGs). Serum antibodies to *Aeromonas* strains and to toxins of *Aeromonas* strains in healthy carriers, in patients with aeromonas-associated diarrhea, and in healthy blood donors were studied.

MATERIALS AND METHODS

Sera and bacteria. Serum specimens were collected from patients with *Aeromonas veronii*-associated diarrhea (n = 11), *A. hydrophila*-associated diarrhea (n = 5), *A. media*-associated diarrhea (n = 1), or *A. caviae*-associated diarrhea (n = 6) and from patients with mixed infections from which an *Aeromonas* strain and a known enteropathogenic species were isolated (n = 3) (Table 1). Also included were serum specimens from healthy individuals with *A. hydrophila* (n = 1), *A. veronii* (n = 1), or *A. caviae* (n = 4) in their feces. The clinical significance and phenotypic characteristics of the *Aeromonas* strains used in this study have been described previously (12–14). *Aeromonas* strains were identified to species level by DNA relatedness studies with the reference strains from the 11 known *Aeromonas* hybridization reference groups, as previously described (15, 23; J. J. Farmer III, F. W. Hickman-Brenner, G. R. Fanning, M. J. Arduino, and D. J. Brenner, Abstr. Int. Workshop *Aeromonas*, Manchester, England, abstr. no. P1, 1986). Serum samples from 50 healthy blood donors (who had no history of recent diarrhea) were used to set up a cutoff level for the enzyme-linked immunosorbent assay (ELISA) and the toxin-neutralizing-antibody assay. In addition, the sera were pooled and used as a reference serum in the ELISA. Convalescent-phase serum from a 54-year-old male with septicemia caused by *A. veronii* was used as a standard positive control serum. Serum from a 1-month-old healthy baby without aeromonads in his feces was included in each assay as a negative control serum.

Isolation of cell envelopes. Cell envelopes were isolated

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TABLE 1. Spectra of illness and HGs of *Aeromonas* isolates from 32 individuals in the serologic study

Group no.[a]	Patient no.	Age	HG of *Aeromonas* isolate[b]	Feces			
				Semi-solid	Watery	With:	
						Blood	Mucus
1	A-2	21 yr	8	−	+	−	−
	A-4	59 yr	8	−	+	−	−
	A-5	27 yr	8	+	−	−	+
	A-10	7 mo	4	+	−	−	−
	A-12[c,d]	82 yr	8	+	−	+	−
	A-19	3 yr	4	+	−	−	−
	A-21	75 yr	8	+	−	−	+
	A-22	78 yr	5A	+	−	−	−
	A-38[c]	63 yr	8	−	−	+	+
	A-39[d]	8 yr	1	+	−	−	+
	A-45[d]	66 yr	8	−	+	+	−
	A-62[c]	3 mo	4	+	−	−	−
	A-87[d]	78 yr	8	+	−	−	+
	A-114[c,d]	80 yr	8	+	−	+	+
	A-179	31 yr	8	+	−	−	+
2	A-15	10 mo	8	+	−	+	+
	A-18	20 yr	4	−	+	−	+
	A-23	42 yr	4	+	−	+	+
	A-33	2 yr	1	−	+	−	−
	A-97	22 yr	1	+	−	+	+
	A-124	36 yr	4	+	−	−	+
	A-160	81 yr	1	−	+	−	−
	A-176	7 yr	1	+	−	−	+
3	A-9	4 mo	4	+	−	−	−
	A-16	27 yr	4	+	−	−	−
	A-26	26 yr	NT	+	−	−	−
	A-34[c]	10 yr	8	+	−	−	−
	A-46	18 yr	4	+	−	−	−
	A-189[c]	18 yr	1	+	−	−	−
4	A-7	15 mo	1	+	−	−	+
	A-8	65 yr	4	−	+	−	+
	A-136	5 yr	4	+	−	−	−

[a] Group 1, Patients with acute gastroenteritis; group 2, patients with chronic gastroenteritis (diarrhea for more than 2 weeks); group 3, healthy controls with *Aeromonas* spp. in their feces; group 4, patients with other enteric infections (*Salmonella typhimurium* [patients A-7 and A-136] and *Campylobacter jejuni* [patient A-8]) and *Aeromonas* spp. in their feces.

[b] HG 1, *A. hydrophila*; HG 8, *A. veronii*; HG 4, *A. caviae*; HG 5A, *A. media*. NT, Not tested.

[c] Only one serum sample was available.

[d] Patient was hospitalized with diarrhea.

(18) by growing *Aeromonas* strains to late logarithmic phase in 30 ml of brain heart infusion broth. The cells were harvested, washed with 15 mM sodium phosphate–0.1 M NaCl (pH 7.4) (phosphate-buffered saline [PBS]), and suspended in 50 mM Tris hydrochloride (pH 7.8). After sonic disruption of cooled cells by four 14-s exposures (sonifier and B15 cell disrupter [Branson Sonic Power Co., Danbury, Conn.]), unbroken cells and large fragments were removed by centrifugation for 20 min at 2,000 × *g*. The supernatant fluid was centrifuged for 60 min at 45,000 × *g*. The pellet containing the envelopes was suspended in 2 mM Tris hydrochloride, pH 7.8. Quantitative measurements of protein in the cell envelopes were done by the method of Lowry et al. (17).

Isolation of LPS and capsular polysaccharides. Lipopolysaccharides (LPS) were extracted and purified by the phenol method as described by Westphal et al. (31), except that the cells were extracted twice. After removal of phenol by ether extraction, LPS were purified by ultracentrifugation (done twice) at 100,000 × *g* for 4 h. LPS were dialyzed for 24 h against deionized water, lyophilized, and stored at −20°C. Capsular polysaccharides were isolated by Cetavlon precipitation, dissociation in 1.0 M $CaCl_2$, phenol extraction, and ultracentrifugation as described by Ørskov et al. (21).

Protein A affinity chromatography of serum. Protein A affinity chromatography was done by conventional methods described in detail previously (27). Immunoglobulin G (IgG)-depleted sera were used for investigations of the IgM response by Western immunoblotting.

Direct bacterial agglutination assay. Serum specimens of patients were tested in duplicate against the homologous *Aeromonas* strains. *Aeromonas* strains were grown for 18 h at 37°C on 10% sheep blood agar plates and were suspended in PBS to a density corresponding to a McFarland standard of 5. Serial twofold dilutions of serum specimens in 50 μl of PBS were mixed with 50 μl of bacterial cells in microdilution V plates (Greiner and Söhn GmbH, Nürtingen, Federal Republic of Germany). PBS instead of serum was used as a control to detect autoagglutination. The plates were agitated gently, sealed with plate sealers, and incubated at 37°C for 2 h and then at 4°C for 18 h. Titers were recorded as the highest serum dilutions showing bacterial agglutination. The agglutination assay was considered positive when the patient had developed a fourfold increase in agglutinating antibodies in the second serum sample, compared with the first serum sample.

Toxin-neutralizing assays. *Aeromonas* strains were grown in 5 ml of brain heart infusion broth for 14 h at 34°C in a shaking water bath. The filtered supernatant was diluted twofold in 1 ml of Eagle minimal essential medium (Flow Laboratories, Irvine, United Kingdom) containing gentamicin (20 μg/ml), penicillin (100 U/ml), and amphotericin B (1 μg/ml). Subsequently, the supernatant was transferred to a 3-day-old monolayer of Vero cells. After incubation for 16 h at 34°C, the cells were examined microscopically, and the cytotoxic titer was defined as the highest dilution that gave distinct degeneration or vacuolization for 50% of the cells. The filtered supernatant was also serially diluted with PBS in microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.). Rabbit erythrocytes were then added to a final concentration of 0.5% (vol/vol). After incubation for 1 h at 37°C and then for 12 h at 4°C, the hemolytic titer was defined as the highest dilution that gave 50% hemolysis. A hemolytic standard control ranging from 10 to 100% hemolysis was included in each assay. Toxin concentrations fourfold higher than the cytotoxic and hemolytic titers were used in the neutralizing tests. Twofold dilutions of sera in PBS (0.5 ml) were incubated with equal volumes of diluted cytotoxic supernatants (prepared from homologous *Aeromonas* strains) for 2 h at 37°C, added to Eagle minimum essential medium and then incubated at 37°C overnight. The highest dilution of serum which neutralized the cytotoxic effects on 50% of the Vero cells was considered the endpoint. A similar method was used for hemolysin-neutralizing activity. Sera containing this neutralizing activity were considered positive. A positive serum control for cytotoxic and hemolytic effects and a negative serum control were included in each test. To test the specificity of the toxin-neutralizing assays, serum specimens from 50 healthy blood donors were investigated for cytotoxin- and hemolysin-neutralizing activities.

Indirect noncompetitive solid-phase ELISA. For ELISA (27), microdilution plates (Greiner) were coated with cell envelopes (10 μg of protein per ml) in 0.1 ml of PBS per well during an overnight incubation at room temperature. After

six washes with PBS containing 0.5% (vol/vol) Tween 20 (PBS-T), the wells were postcoated with 3% bovine serum albumin (radioimmunoassay grade; Sigma Chemical Co., St. Louis, Mo.) in PBS for 2 h at 37°C and washed three times with PBS-T. Serum dilutions were prepared serially from 1:200 to 1:25,600 in PBS, and 0.1 ml of each serum dilution was added to duplicate wells of vertical rows in the microdilution plates. Serum samples from each patient, a pooled serum from 50 healthy blood donors, a positive control serum, and a negative control serum were tested against cell envelopes of the bacterial strains of the same patient. After incubation for 2 h at 37°C, the microdilution plates were washed six times with PBS-T, and then conjugate (goat anti-human IgG, gamma chain specific, labeled with horseradish peroxidase; Tago Inc., Burlingame, Calif.) was added at the optimal dilution (as indicated by the manufacturer) in PBS-T containing 0.5% bovine serum albumin. After incubation for 3 h at 37°C and six washes with PBS-T, 0.1 ml of substrate solution was added to each well. The substrate solution was prepared just before use and consisted of 0.02 M *ortho*-phenylenediamine (BDH Chemicals Ltd., Poole, United Kingdom) and 0.01% H_2O_2 in 50 mM phosphate–25 mM citric acid (pH 5.0). After incubation for 20 min in the dark at room temperature, the reaction was stopped by adding 30 μl of 50% H_2SO_4 to each well. Absorbance was read at an optical density of 492 nm on a Titertek Multiskan (Flow Laboratories). Sera were tested in duplicate, and the antigen-coated control well reading was subtracted from the mean optical density of the sera. The titer of each serum was extrapolated from the ELISA curve as the dilution which gave an optical density of 1.0. The absorbance value (optical density = 1) was always in the linear part of the sigmoid dose-response curves. To correct for differences in antigenic composition of the strains, titers were expressed relative to those of human pooled serum with the same antigen. Pooled serum of samples from 50 healthy donors served to calculate the titer of the negative group (N value), whereas the tested serum yielded the P value. Sera were considered positive if P/N ratios were greater than or equal to 1.8 (mean of the negative sera plus 2 standard deviations; see Results).

GIRA. Gel immunoradioassay (GIRA) was done by a modification of the method of Poolman and Zanen (22). Cell envelopes of *Aeromonas* strains were suspended to a final concentration of 1 mg of protein per ml in a buffer containing 62.5 mM Tris hydrochloride (pH 6.8), 2% (wt/vol) sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue and were boiled for 5 min. SDS-polyacrylamide gel electrophoresis was done by the method of Lugtenberg et al. (18) with concentrations of 12% acrylamide in the running gel and 5% acrylamide in the stacking gel. Boiled samples (20 μl) were applied to the gel. After electrophoresis for 3 h at 30 mA, SDS gels were frozen in liquid nitrogen and cut into 50-μm slices (5 by 5 cm) at −35°C with a 5030 microtome (Bright Instrument Co. Ltd., Huntingdon, United Kingdom). Gel slices were stored at −20°C in ethanol-acetic acid (6:1) until use. Before use, the slices were washed with PBS and incubated with patient serum (1:10, diluted in PBS) for 30 min at room temperature and overnight at 4°C. After three washes with PBS at room temperature, the slices were incubated with ^{125}I-protein A in PBS containing 0.5% bovine serum albumin and 0.01% Tween 80 for 2 h at room temperature, with 10^6 cpm per slice. After three washes with PBS (16 h each) at room temperature, the slices were autoradiographed on X-Omat-R film (Eastman Kodak Co., Rochester, N.Y.) at −70°C with one intensifying screen.

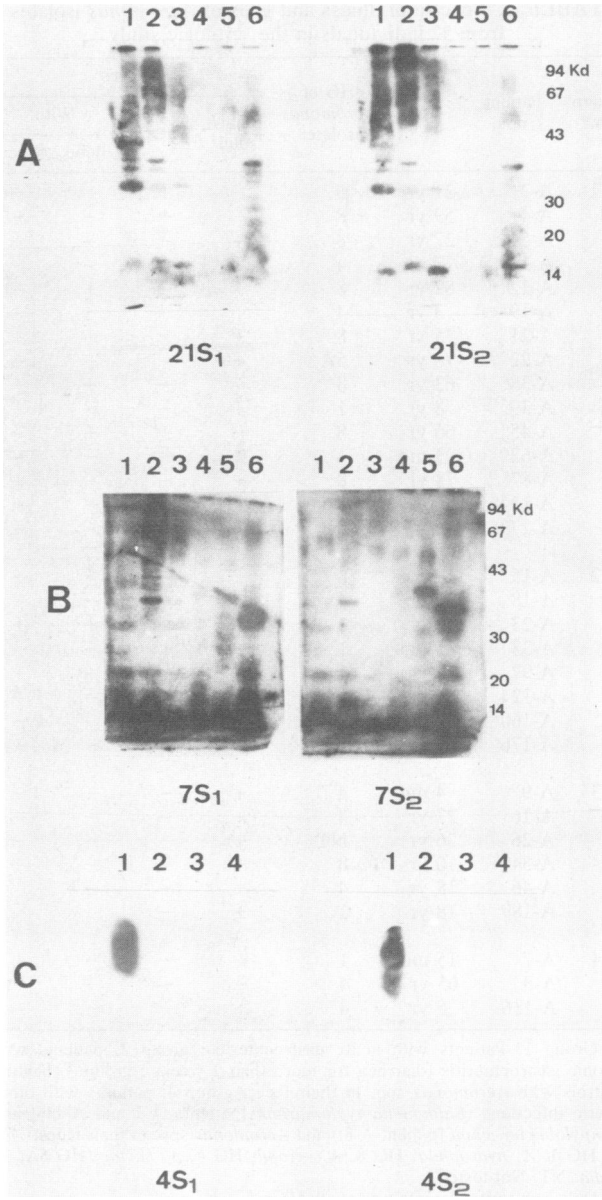

FIG. 1. First serum samples (S_1) and second serum samples (S_2) from patients A-21, A-7, and A-4 as examined by GIRA for antibodies against cell envelopes (A and B) and purified LPS (C). Kd, Kilodaltons. (A) Lane 1, Homologous *Aeromonas* strain A-21; lane 2, *Aeromonas* strain A-7; lane 3, *Aeromonas* strain A-18; lane 4, *P. aeruginosa*; lane 5, *V. cholerae*; lane 6, *E. coli* O3:K4. (B) Lane 1, *Aeromonas* strain A-21; lane 2, homologous *Aeromonas* strain A-7; lane 3, *Aeromonas* strain A-16; lane 4, *P. aeruginosa*; lane 5, *V. cholerae*; lane 6, *E. coli* O3:K4. (C) Lane 1, Homologous *Aeromonas* strain A-4; lane 2, *Aeromonas* strain A-18; lane 3, *Aeromonas* strain A-22; lane 4, *E. coli* O3:K4.

Slices not incubated with sera were stained with silver or Coomassie blue so the nature of the antibody response could be studied more specifically.

Western immunoblotting. For Western immunoblotting (28), cell envelopes of *Aeromonas* strains were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BA85, 0.45-μm pore size; Schleicher & Schuell, Dassel, Federal Republic of Germany) in 25 mM Tris hydrochloride–192 mM glycine (pH 8.3) blotting

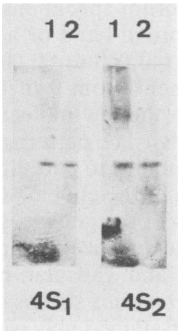

FIG. 2. First (S_1) and second (S_2) serum samples from patient A-4 as examined by Western immunoblotting for IgM antibodies against the homologous *Aeromonas* strain A-4 (lanes 1) and *Aeromonas* strain A-33 (lanes 2).

buffer containing 20% methanol and 0.1% empigen at 25 V for 18 h. To prevent nonspecific absorption, blots were then incubated in a blocking buffer of PBS with 5% heat-inactivated goat serum. After the blots were incubated for 2 h at room temperature, serum dilutions (1:50) were added to them and the blots were subsequently incubated overnight at room temperature. The blots were then rinsed 10 times for 10 min each with PBS-T and reincubated in blocking buffer for 30 min at room temperature before conjugate (goat anti-human IgM, mu chain specific, labeled with horseradish peroxidase; Tago Inc.) was added to the buffer solution. After incubation for 2 h at room temperature, the blots were washed 10 times for 10 min each with PBS-T, and substrate was added to visualize the blots. The substrate solution was freshly made and consisted of 10 ml of 18 mM dioctyl sulfosuccinate and 10 mM tetramethylbenzidine in 96% ethanol, 30 ml of 5 mM citric acid–10 mM Na_2HPO_4 (pH 5.0), and 20 μl of 30% H_2O_2. The staining reaction was stopped by rinsing the blot with water for 1 min and washing it for 10 min in 0.2% dioctyl sulfosuccinate–25% ethanol. Blots were dried at room temperature in the dark. The antibody response was compared with that on silver- and Coomassie blue-stained gels of the same *Aeromonas* cell envelopes.

RESULTS

Patients and sera. Patients with aeromonads in their feces were grouped into four categories according to clinical symptoms and to the presence of other enteropathogenic bacteria (Table 1). The mean ages were 48 years for group 1, 26 years for group 2, 17 years for group 3, and 28 years for group 4. The occurrence of various DNA HGs differed in frequency in two of the categories: 10 of 12 strains belonging to HG 8 were cultured from feces of patients with acute gastroenteritis (group 1), whereas 4 of 7 strains from HG 1 occurred in the group of patients with chronic gastroenteritis (group 2). HG 1 is *A. hydrophila*, while HG 8 is *A. veronii* (which has been reported as *A. sobria* by clinical laboratories [15]). HG 4 is *A. caviae*, and HG 5A is *A. media*.

Sera were collected as soon as bacteriological results from fecal cultures were known. The mean intervals between the onset of the gastrointestinal symptoms and collection of the first serum sample were 9.6 days for group 1 (range, 2 to 30 days), 21.5 days for group 2 (range, 14 to 31 days; patient A-97 had an interval of 90 days), and 10.7 days for group 4 (range, 8 to 14 days). The interval between culture of the fecal sample and collection of the first serum sample from healthy controls in group 3 was 8.4 days (range, 5 to 14 days; patient A-189 had an interval of 180 days).

Bacterium-agglutinating antibodies. Because of the great variation in profiles of proteins and polysaccharides from cell envelopes examined by SDS-polyacrylamide gel electrophoresis, agglutination assays were done with the homologous *Aeromonas* strain as antigen (Ed J. Kuijper and M. F. Peeters, Abstr. Int. Workshop *Aeromonas*, Manchester, England, abstr. no. P15, p. 15–16, 1986). Autoagglutination of bacteria occurred when Formalin-killed bacteria were used as antigen. We therefore used living bacteria as antigen. When serum specimens from 10 healthy blood donors (from the pool of 50 donors) were tested against 10 different *Aeromonas* strains, agglutination titers ranged from 1:10 to 1:640 without any clear pattern. Similar strain-specific variations in agglutination titers were observed in serum specimens from 32 individuals with aeromonads. One patient (A-21) was hospitalized for claudication and developed acute aeromonas-associated diarrhea with a fourfold increase in serum-agglutinating antibody titer against the homologous strain during his hospital stay.

ELISA. Serum specimens from 50 healthy blood donors were tested against four *Aeromonas* strains from different HGs (strains from patients A-7, A-18, A-22, and A-38) to determine the mean values of negative sera and the specificity of the ELISA. The mean P/N ratios plus standard deviations were 0.71 ± 0.47 (A-18), 0.74 ± 0.53 (A-38), 0.73 ± 0.45 (A-7), and 0.68 ± 0.50 (A-22). P/N ratios were considered positive if they were two standard deviations above the mean of the negative control sera (95% confidence interval). Subsequently, positive ratios were greater than or equal to 1.80. This cutoff value was also based on the lowest value of the positive control serum, which ranged from 2.06 to 56.80, depending on the *Aeromonas* strain used as antigen. The P/N ratios of the negative reference serum ranged from 0.18 to 0.84. By using the cutoff value of 1.80 for patients with acute or chronic aeromonas-associated diarrhea, the sensitivity of the ELISA was 30% (7 of 23 patients were positive; Table 2), whereas the specificity was 74% (13 of 50 healthy blood donors were positive). With regard to the specificity, 11 of 50 serum specimens were positive against strains from A-7 and A-22, 13 were positive against strains from A-18, and 9 were positive against strains from A-38. Of the 11 specimens, 6 were positive against all four strains tested. However, all patients with mixed infections ($n = 3$) and three (50%) of six healthy individuals with aeromonads in their feces also showed positive P/N ratios (Table 2). When an age-matched control group of six healthy individuals with fecal cultures negative for *Aeromonas* spp. (five blood donors and one baby) was tested against *Aeromonas* strains from the same HGs as those isolated from six healthy carriers, none of the serum specimens were positive.

Neutralizing antibodies against cytotoxins and hemolysins. Serum specimens from 50 healthy blood donors were tested for cytotoxin- and hemolysin-neutralizing activities. An *Aeromonas* strain from patient A-87 (HG 8) was used to prepare cytotoxins and hemolysins. A total of 3 (6%) of 50 serum specimens showed cytotoxin-neutralizing activity at low titers of 1:4 (2 specimens) and 1:8 (1 specimen). None of the 50 serum specimens neutralized hemolysins.

Nineteen patients with toxin-producing *Aeromonas* strains were examined for the presence of serum-neutralizing antibodies against cytotoxins and hemolysins of the homologous strain. *Aeromonas* strains from six patients (A-4, A-5, A-7, A-34, A-45, and A-189) produced only cytotoxins and no detectable hemolysins. The control serum

TABLE 2. Positive results from ELISA and serum-neutralizing-antibody assay

Group no.[a]	Patient no.	ELISA results[b]		Serum-neutralizing antibodies			
				S_1		S_2	
		S_1	S_2	Cyto[c]	Hemo[d]	Cyto	Hemo
1	A-2	2.11	1.87	<4	<4	<4	<4
	A-4	15.13	9.60	<4	—	<4	—
	A-12	3.68	—[e]	640	160	—	—
	A-21	2.63	2.00	640	—	640	—
	A-38	1.22	—	160	160	—	—
	A-87	0.69	0.92	<4	4	160	40
	A-114	1.48	—	40	40	—	—
2	A-15	2.02	2.25	<4	<4	<4	<4
	A-18	2.65	3.40	<4	<4	<4	<4
	A-124	2.06	1.85	<4	<4	<4	<4
3	A-26	2.00	0.98	—	—	—	—
	A-34	2.23	—	<4	—	—	—
	A-46	2.06	0.92	—	—	—	—
4	A-7	3.37	1.35	<4	—	<4	—
	A-8	2.06	2.13	—	—	—	—
	A-136	0.94	3.50	—	—	—	—

[a] Group 1, Patients with acute gastroenteritis; group 2, patients with chronic gastroenteritis (diarrhea for more than 2 weeks); group 3, healthy controls with aeromonads in their feces; group 4, patients with other enteric infections and aeromonads in their feces.

[b] Expressed as P/N ratios (see text). Sera were considered positive when P/N ratios were greater than or equal to 1.80. S_1 and S_2, First and second serum samples, respectively.

[c] Antibodies against cytotoxin to Vero cells. Results are expressed as the reciprocal of the titer.

[d] Antibodies against hemolysin to rabbit erythrocytes. Results are expressed as the reciprocal of the titer.

[e] —, Not tested. The *Aeromonas* strain did not produce hemolysin or cytotoxin, or only one serum sample was available.

of a patient with septicemia caused by a toxin-producing *Aeromonas* strain did not contain neutralizing antibodies. A total of five (26%) of 19 patients had neutralizing antibodies at titers greater than 1:4 (Table 2). All five patients suffered from severe acute gastroenteritis, and three were hospitalized because of diarrhea. In one of these five patients (A-87), seroconversion against the cytotoxin and a 10-fold rise in titer against the hemolysin were observed. Five (83%) of six patients older than age 60 who had acute diarrhea responded to cytotoxins, whereas 0 (0%) of five patients younger than age 60 who had acute diarrhea responded ($\chi^2 = 4.65$ [including Yates correction], $P < 0.05$).

The positive serum samples of two patients (A-38 and A-12) were tested against cytotoxins and hemolysins in reciprocal reactions. The titers were identical to those of the toxins from the homologous strain.

GIRA. Paired serum specimens from 19 patients (8 patients from group 1, 7 from group 2, 2 from group 3, and 2 from group 4) were examined by GIRA for antibodies against cell envelopes of the homologous and heterologous *Aeromonas* strains, an *Escherichia coli* (O3:K4) strain, a *Pseudomonas aeruginosa* strain, and a *V. cholerae* strain. Serum specimens from 10 patients (3 from group 1, 5 from group 2, and 2 from group 4) reacted with components from cell envelopes of the homologous *Aeromonas* strain. Serum specimens from 8 of these 10 patients also reacted with *Aeromonas* strains from other HGs (Fig. 1A). Strains from identical HGs did not contain common antigens that were specific for the HG. Serum specimens from the 10 patients reacted with either a broad-band or a ladder pattern in the high-molecular-weight (40,000 to 80,000) regions of the gel, whereas serum specimens from 9 of the 10 patients had a broad smear present in the low-molecular-weight (10,000 to 25,000) fraction (Fig. 1B). The patterns at the front of the gel were indicative of antibodies to core-lipid A when compared with silver-stained gels of the envelopes. Since the nature of the reactions at the top of the gel was suggestive of antibodies to LPS with long polymerized O antigens or to capsular polysaccharides, LPS and capsular polysaccharides were isolated from three *Aeromonas* strains of different HGs (A-4, A-18, and A-22). Silver-stained gels were used to detect the presence of LPS and to exclude rough isolates or contamination with proteins or nucleic acids. The antibodies of the three tested serum specimens appeared to be directed against the LPS fraction. Patient A-4 was of special interest, since she developed a strong reaction to the O antigen and the core-lipid A fraction of the homologous *Aeromonas* strain without cross-reactivity to heterologous strains (Fig. 1C; the core and lipid A fraction is not present on this gel). ELISA results for sera from patient A-4 were also positive, whereas the toxin-neutralizing assay was negative.

Antibodies also reacted with proteins of homologous and heterologous cell envelopes when GIRA results were compared with the results on Coomassie blue-stained gels of the cell envelopes, but these reactions did not consistently show a clear common pattern. No common specific antigens were found among strains from identical HGs. Serum specimens from 18 of 19 patients demonstrated antibodies against polysaccharides or proteins from cell envelopes of *E. coli* but not of *P. aeruginosa* or *V. cholerae*. The antibody response against *E. coli*, however, was always weaker than the antibody response to *Aeromonas* strains. Serum specimens from five healthy blood donors were also tested for antibodies against *Aeromonas* spp. by GIRA, and all sera showed some weak reactivity to LPS and proteins from cell envelopes of *Aeromonas* strains.

Western immunoblotting. Sera from seven patients (A-4, A-8, A-10, A-22, A-23, A-33, and A-45) were examined for IgM antibodies against homologous strains by Western immunoblot analysis. Two paired serum specimens (A-4 and A-23) showed positive reactions. When the immunoblotting results were compared with the results on Coomassie blue- and silver-stained gels, the reaction looked like an antibody response to LPS. Serum specimens from patient A-4 reacted only with LPS of the homologous *Aeromonas* strain (Fig. 2).

DISCUSSION

The genus *Aeromonas* contains at least 11 HGs that are difficult, or in some cases impossible, to phenotypically separate from each other (15, 23). Strains belonging to certain HGs produce enterotoxins, cytotoxins, and hemolysins, but it is not clear if these toxins result from different molecules or the same molecule (16). Some studies report a good correlation between enterotoxin production and hemolysin production, and other studies show that hemolysins of *Aeromonas* spp. are also cytotoxic to tissue-cultured cell lines (16, 29). Recently, Kozaki et al. found that the hemolytic and enterotoxigenic activities of *A. sobria* are neutralized by a monoclonal antibody against the hemolysin, which suggests that one molecule acts as both enterotoxin and hemolysin (11). The discrepancy that we noticed between the production of hemolysins and that of cytotoxins in 6 of 19

Aeromonas strains may be explained by the insensitivity of rabbit erythrocytes to hemolysins of *Aeromonas* strains (3). Another possibility may be the requirement of trypsin activation of the cell-free hemolysin for maximal expression (11). This led us to use separate neutralizing assays for hemolysins and cytotoxins, but it remains possible that some neutralizing antibodies neutralize a hemolysin which also has cytotoxic activity. The results from our previous study (12) showed that noncytotoxigenic *Aeromonas* strains belonging to HG 4 and HG 5A were isolated more frequently from human feces (67%) than were cytotoxigenic (to Vero cells) strains belonging to HGs 1, 2, 3, and 8 (33%). In addition, strains in HG 8 were cultured predominantly from patients with acute gastroenteritis, whereas strains in HG 1 were cultured from patients with chronic diarrhea. Thus, a different distribution of HGs existed in the four categories of patients that we have included in this study. The occurrence of HGs was age related, which also resulted in a different age distribution among the four categories.

The results from this study indicate that elderly patients with acute gastroenteritis may develop a serum antibody response to cytotoxins and hemolysins of *Aeromonas* strains. The specificity of the toxin-neutralizing assay was 94%, as determined by testing the serum specimens of 50 healthy blood donors. Toxin-neutralizing antibodies were present at high titers in 5 (46%) of 11 patients who had developed acute severe diarrhea associated with *Aeromonas* strains predominantly from HG 8. Interestingly, three of these five patients had bloody diarrhea. The six patients who did not respond to cytotoxins or hemolysins were not affected by immunosuppression from medication or underlying diseases. Only one of these six patients had bloody diarrhea. In addition, the feces of patient A-87 (who had developed a significant rise in toxin-neutralizing antibodies in serum) were examined for the presence of free fecal cytotoxins (12). The stool extract of patient A-87 contained cytotoxins which could not be neutralized by *Clostridium sordellii* antitoxins and were suggestive of *Aeromonas* cytotoxins. However, neutralization with anti-Shiga toxin antibodies to exclude Shiga-like toxins was not performed.

All five patients who responded to hemolysins or cytotoxins were above the age of 60 and three were hospitalized because of severe diarrhea, suggesting that only severe damage to the intestinal tract will give rise to neutralizing antitoxins. This hypothesis is strengthened by two published case reports on hospitalized patients with severe choleralike diarrhea who showed a rise in neutralizing antibody to *Aeromonas* toxins (5, 9). Cross-reacting antigens, such as cholera toxin and perhaps enterotoxins from *E. coli* or toxins from unidentified bacteria, however, could theoretically have produced serum antibodies in our patients (6, 26). To establish the specificity of the neutralizing assay more carefully, further testing with cytotoxins and hemolysins from other bacterial species is warranted.

The predominance of noncytotoxigenic *Aeromonas* isolates of HG 4 and HG 5A in human feces prompted us to investigate the human serum antibody response to surface components of *Aeromonas* strains by ELISA and bacterial agglutination. Agglutination was used by Caselitz et al., who described two patients with significant rises in serum-agglutinating antibodies against homologous *Aeromonas* strains: one patient had a chronic leg ulcer, and the other suffered from a liver abscess (4). We used homologous *Aeromonas* strains as antigens, because the genus *Aeromonas* is serologically heterogeneous in O antigens and H antigens and because of the great variation in protein profiles observed when the cell envelopes were examined by SDS-polyacrylamide gel electrophoresis (Kuijper and Peeters, Abstr. Int. Workshop *Aeromonas*, 1986). Evaluation of the bacterial agglutination assay showed that it was not very useful.

An ELISA was also done with homologous *Aeromonas* strains as antigen. Sera from 50 healthy blood donors were used to set up a cutoff value for the ELISA. The sensitivity (30%) and the specificity (74%) of the ELISA were disappointing, however. The results of the ELISA did not reveal differences between serum specimens from the four patient groups tested. A total of 3 (50%) of 6 individuals without gastrointestinal symptoms developed an antibody response to *Aeromonas* strains, which indicates that results from the ELISA do not discriminate between aeromonas-associated diarrhea and asymptomatic colonization. GIRA results suggested that cross-reactions in ELISA may be due to preexisting antibodies not only to *Aeromonas* strains but also to *E. coli*. Fecal samples containing known enteropathogenic bacteria in addition to aeromonads also gave rise to serum antibody responses against *Aeromonas* strains. A poor correlation was found between ELISA results and toxin-neutralizing assays, since only two of five patients who developed neutralizing antibodies were positive in the ELISA. When the patient group with mixed infections was excluded, positive ELISA results were observed in patients with *Aeromonas* strains in HG 8 (50% positive) and HG 4 (17% positive) in their feces. Patients with positive fecal cultures of *Aeromonas* strains in HG 1 ($n = 6$) were negative in the ELISA and in bacterial agglutination and toxin-neutralizing assays. This is an interesting observation, since *Aeromonas* strains in HG 1 were cultured predominantly from patients with chronic diarrhea.

The results of bacterial agglutination assays and ELISA supported the concept of immunological variation and relatedness among *Aeromonas* strains. When examined qualitatively by GIRA (IgG) and Western immunoblotting (IgM), antibodies were most frequently directed against the O antigens of homologous *Aeromonas* strains, but also against some heterologous strains. The IgG antibodies also showed some cross-reaction with polysaccharides from *E. coli*, although this response was weaker than that with *Aeromonas* strains. No common specific antigens were found among strains from identical HGs. Sakazaki and Shimada reported at least 44 O antigens in the mesophilic *Aeromonas* species, some of which are immunologically related to O antigens of *Vibrio* species and *Plesiomonas shigelloides* (24). Studies of the association of gastroenteritis and the occurrence of LPS with O polysaccharides of *Aeromonas* spp. have not been done and may be of particular interest for non-toxin-producing *Aeromonas* strains.

In summary, of three different serologic assays developed to measure serum antibody response against intestinal *Aeromonas* strains, only the toxin-neutralizing assay distinguished patients with acute gastroenteritis from other groups of patients and from healthy blood donors. To study the diagnostic value of the toxin-neutralizing assay more carefully, further research is necessary to determine the antigen specificity and to increase the sensitivity of the assay.

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