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Immune Response to *Escherichia coli* Alpha-Hemolysin in Patients

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The serum antibody response of patients infected with alpha-hemolysin (AH)-producing *Escherichia coli* was measured by three immunoassays: tube neutralization, microneutralization, and enzyme-linked immunosorbent assay. All three assay results showed good correlation with each other. The mean anti-AH titer in patients with *E. coli* infection was higher than the mean titer in noninfected patients. The hemolysin-neutralizing activity was immunoglobulin G. The amount of lipopolysaccharide (LPS) antibody did not correlate with the amount of AH antibody. LPS antibody measured by enzyme-linked immunosorbent assay was predominantly of the immunoglobulin G class. Adsorption of LPS antibody by *E. coli* H79 LPS did not affect anti-AH titers, indicating that LPS and AH have different antigenic determinants. AHs prepared from several different *E. coli* strains had identical or similar antigenic determinants at the active site. Hemolysin proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were stained identically by human sera with AH antibody and by a mouse monoclonal AH antibody.

Hemolytic *Escherichia coli* produces two hemolysins, a cell-bound (beta) hemolysin and a cell-free (alpha) hemolysin (AH) (20). Both hemolysins cause beta-hemolysis on blood agar plates. Hemolysin is an important virulence factor in extraintestinal infections and is associated with urinary tract infection (UTI), bacteremia, peritonitis, and septicemia (6, 8, 13). DNA hybridization probe studies done by O'Hanley et al. (19) showed that the galactose-galactose-binding adhesin and hemolysin are two major determinants of virulence in uropathogenic *E. coli*. Studies on the effects of hemolytic *E. coli* in animals indicate that hemolysin contributes to the severity of experimental pyelonephritis (9, 10, 23, 24). *E. coli* AH may induce renal tubular cell injury in vitro by generating oxygen intermediates like H_2O_2 and by depleting ATP (15). Cavalieri and Snyder showed that AH (purified by glycerol gradient) is cytotoxic for mouse fibroblasts and human peripheral leukocytes (3–5).

AH is antigenic in both humans and animals. AH-neutralizing antibodies are found in patients with hemolytic *E. coli* infections, and titers appear to correlate with the severity of infection (16, 20). Antibody titers also correlate with the invasiveness of the infection, i.e., pyelonephritis > cystitis > asymptomatic bacteriuria (7). Recent studies show that monoclonal antibody to AH prevents hemolysis and cytotoxicity for human peripheral leukocytes (1).

The purpose of our study was to confirm and extend the studies on antibody response of humans to AH as reported by others (7, 16, 20) and to investigate some of the factors that might affect measurement of the antibody response.

MATERIALS AND METHODS

AH production and measurement. *E. coli* H79, a strain isolated from a human UTI, was used for hemolysin production (1, 3). Organisms were grown in a chemically defined medium described previously (21). Crude hemolysin concentrate was prepared by the method described by Cavalieri and Snyder (5). Hemolytic activity was determined by using a modification of the method described by Snyder and Koch (21). Serial twofold dilutions of the hemolysin preparation were made in 1 ml of calcium saline containing 10 mM $CaCl_2$. A 1-ml portion of a standard 1% sheep erythrocyte (SRBC) suspension was added to all the tubes, and the contents were incubated for 1 h at 37°C. Unlysed erythrocytes were removed by centrifugation after the addition of 2 ml of cold saline to each tube. The A_{545} of each decanted supernatant was measured. Fifty percent lysis of the standard 1% SRBC suspension was used as the endpoint. The hemolysin titer was extrapolated from a standard curve by plotting A_{545} versus the $\log_{10}$ of the dilution on a semi-logarithmic graph paper and expressed as HU_{50} per milliliter. One HU_{50} of hemolysin lyses 50% of the standard SRBC suspension and gives an optical density of 0.3 at 545 nm.

Sera. Sera were obtained from patients at St. Josephs Mercy Hospital, Ann Arbor, Mich., and from Community General Hospital, Syracuse, N.Y. They were divided into an infection group and a control group. The control group consisted of 20 patients without hemolytic *E. coli* infections. Fifty-three patients in various stages of infection with hemolytic *E. coli* formed the infection group. The serum samples from these patients were collected on diagnosis of UTI.

Antibody assay. (i) Tube neutralization assay. Each serum was diluted from 1:100 to 1:12,800 to give a final volume of 1 ml in each tube. Three HU_{50} of AH was added to each tube. The tubes were shaken and preincubated in a 37°C water bath for 10 min. A 1% SRBC suspension (1 ml) was added to all the tubes. The tubes were shaken and incubated for 60 min in a 37°C water bath. Hemolysis was measured as described above. The highest dilution of antibody that neutralized 2 HU_{50} of hemolysin was used as the endpoint. The A_{545} of each serum dilution supernatant versus the serum dilution was plotted on a semi-logarithmic scale. The exact dilution that resulted in an A_{545} of 0.3 (indicating neutralization of 2 HU_{50}) was determined from the plot. Controls for AH activity in the absence of antibody and spontaneous hemolysis of the SRBC were included.

(ii) Microneutralization assay. A 100-μl sample of diluted

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serum (1:100 to 1:12,800) was added to separate wells of microtiter plates (96-well U-bottom type; NUNCLON; Intermed; Hazelton Research Products, Inc., Denver, Pa.). Each well then received 100 μl of AH (3 HU_{50}). After incubation of the wells at 37°C for 10 min on a vertical vibrator shaker (Arthur H. Thomas Co., Philadelphia, Pa.), 50 μl of a 2.5% SRBC suspension was added to each well. The plate was shaken continuously at 37°C for 2 h. Unlysed erythrocytes were pelleted by centrifugation at 1,650 × *g*, and 100 μl of each supernatant was transferred to a fresh plate. The A_{570} of each supernatant was measured on a MicroELISA Auto Reader (MR580; Dynatech Laboratories, Inc., Alexandria, Va.). The neutralizing antibody titer was determined as described above for the tube neutralization (TN) assay.

(iii) Enzyme-linked immunosorbent assay. A modification of the direct enzyme-linked immunosorbent assay (ELISA) described by Gustafsson et al. (11) was used to measure AH and lipopolysaccharide (LPS) antibody. *E. coli* H79 LPS was purified by the procedure of Westphal and Jann (25). Test antigens, LPS (25 μg/ml) or crude AH (100 HU_{50}/ml), were diluted in a carbonate coating buffer (0.59 g of Na_2CO_3, 2.92 g of $NaHCO_3$, 0.2 g of NaN_3, water as needed to 1 liter, pH 9.6), added to polyvinyl 96-well assay plates (Costar, Cambridge, Mass.), and incubated at 4°C overnight. Bovine serum albumin was used to block the excess binding sites, and 0.01 M phosphate-buffered saline with 0.5% Tween 20 was used as the wash buffer. Alkaline phosphatase-labeled goat anti-human conjugates (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) directed against immunoglobulin G (IgG) (γ), IgM (μ), and IgA (α) immunoglobulins were used to measure the three classes of AH and LPS antibodies in patient sera. *p*-Nitrophenol phosphate (Sigma Chemical Co., St. Louis, Mo.) was used as the substrate, and the A_{405} of each well was measured on the Dynatech MR580 MicroELISA Auto Reader. An A_{405} of 0.1 at 30 min of incubation was taken as the endpoint of the assay.

ELISA inhibition. A modification of the ELISA inhibition assay as described by Morse and Apicella (18) was used to adsorb the anti-LPS antibodies from patient sera. A stock solution containing 250 μg of *E. coli* H79 LPS per ml (as described above) was made in 0.01 M sodium-phosphate-buffered saline (pH 7.2). Sera positive for anti-LPS antibody were diluted 1:50 in 0.01 M sodium-phosphate-buffered saline (pH 7.2). Equal volumes of LPS stock solution and diluted sera were mixed in 1.8-ml Eppendorf centrifuge tubes (VWR Scientific, Pittsburgh, Pa.) and incubated at 37°C for 1 h and then overnight at 4°C. The tubes were then centrifuged, and serial dilutions were made of each supernatant. Residual anti-LPS and anti-AH activity was measured by ELISA as described above.

SDS-polyacrylamide gel electrophoresis. Crude AH samples were electrophoresed in 5 to 15% gradient polyacrylamide gels by a modification of a method of Laemmli (17). One to two volumes of hemolysin concentrate was added to 20 μl of sample buffer (21% glycerol, 2.1% sodium dodecyl sulfate [SDS], 42% 2-mercaptoethanol, 0.11% bromphenol blue in 0.263 M Tris buffer, pH 6.8) and denatured in a boiling-water bath for 7.5 min. Samples were electrophoresed at 15 mA (constant current) per gel through a 5% polyacrylamide stacking gel (1.5 by 140 by 30 mm) and a vertical slab resolving gel (1.5 by 140 by 100 mm) consisting of a 5 to 15% polyacrylamide gradient. Gels were stained in a 0.2% solution of Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.). Reagents for gel preparation and sample treatment were obtained from Bio-Rad Laboratories.

Immunoblotting. Electrophoresed AH proteins were transferred to 0.2-μm-pore-size nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.). Electrophoretic transfer was accomplished overnight in a model TE-52 transfer unit (Hoefer Scientific Instruments, San Francisco, Calif.) at 4°C with 200 mA (anodal migration) in 0.025 M Tris buffer (pH 8.3) containing 0.19 M glycine and 20% methanol. Transfer efficiency was confirmed by staining some lanes with 0.1% amido black for total protein visualization. The rest of the lanes were incubated in 0.02 M Tris-buffered saline (pH 7.5) (TBS) containing 5% bovine serum albumin (TBS-bovine serum albumin) for 60 min at room temperature. Human sera with high, intermediate, and low anti-AH titers were diluted 1:50 in TBS-bovine serum albumin to give three separate antibody probe solutions. Lanes were separately incubated in the three probe solutions for 90 min and then washed four times (10 min each) in a bath of TBS. Reactivity of the anti-human AH with the AH proteins was detected by incubating the probed lanes with anti-human IgG conjugated to horseradish peroxidase (Kirkegaard and Perry). After washing (as above), the membranes were transferred to a peroxidase substrate solution containing 60 mg of 4-chloro-1-naphthol (Sigma), 60 μl of 30% H_2O_2 (Fisher Scientific Co., Pittsburgh, Pa.), 20 ml of methanol, and 100 ml of TBS for detection of horseradish peroxidase-conjugated antibody.

Patients. The population in the clinical correlation study (average age, 66 years) was divided into a control group and an infection group. Twenty subjects with no history or clinical evidence of UTI were included in the control group. The infection group included 53 subjects with evidence of a hemolytic *E. coli* infection based on urine analysis and culture and blood culture results. Clinically, these patients had a broad range of symptoms varying from a mild cystitis to a severe disseminated infection. Six patients in this group had UTI secondary to immunocompromising conditions. Group comparisons were estimated by the Mann-Whitney U test.

Serial serum samples were obtained from three patients. Patient A, an 84-year-old woman, was admitted with UTI and *E. coli* septicemia. Patient B, an 89-year-old man, had cystitis secondary to bladder catheterization. Patient C, a 69-year-old woman, also had cystitis.

RESULTS

Comparison of immunoassays. Results obtained by the microneutralization (MN) assay and the ELISA were compared with those obtained by the TN assay. The MN assay showed a good correlation with the TN assay (Spearman's rank correlation of 0.93) (Fig. 1). The ELISA results gave a Spearman's rank correlation of 0.83 when compared with the TN assay (Fig. 2). In comparison with the MN and TN assays, the ELISA showed the highest titers (Fig. 2). Serum samples showed high anti-AH IgG titers and low IgM and IgA titers as measured by the three assays (data not shown). The high titers of anti-AH IgG indicate that the AH-neutralizing antibody is IgG (Table 1).

A statistical analysis of the reproducibility of data by ELISA and TN assay showed that a 16% difference in titers by ELISA and a 10% difference by the TN assay were not significant.

Anti-LPS ELISA and LPS inhibition. A total of 70% of the

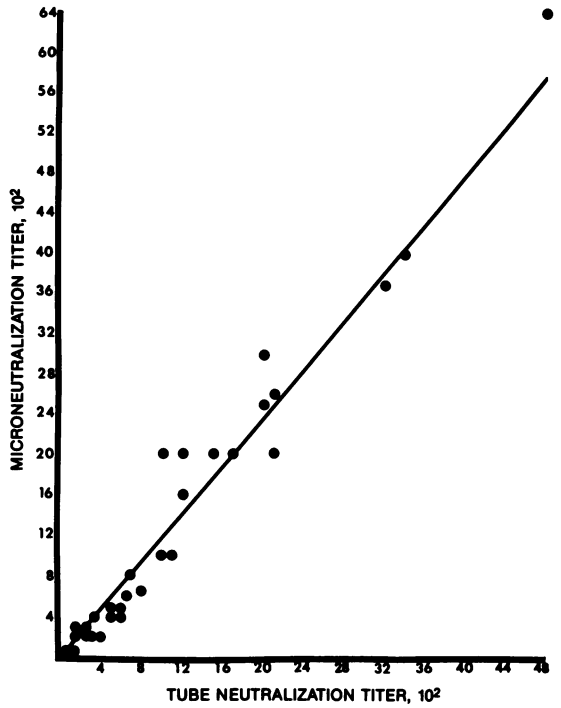

FIG. 1. Correlation between TN and MN assays in the measurement of AH antibody.

samples with AH antibody also contained LPS antibody when tested against *E. coli* H79 LPS. There appears to be no correlation between anti-LPS and anti-AH titers in a given patient (data not shown).

Anti-*E. coli* H79 LPS titers measured by a standard ELISA were compared with anti-AH titers before and after adsorption with LPS (Table 1). The LPS antibody was predominantly IgG. Adsorption of LPS antibody with H79 LPS resulted in adsorption of IgM and IgA without affecting the titer of anti-AH IgG, further confirming that the AH-neutralizing antibody is IgG. Comparing adsorbed sera with unadsorbed sera in the TN assay also showed that adsorption by LPS has no effect on the anti-AH titer (Table 2). These data confirm that LPS and the hemolysin protein are distinct.

Relationship between hemolysin production in vitro and antibody titer. Hemolytic *E. coli* cultures isolated from patients with high and low antibody titers were grown in chemically defined medium and assayed for hemolysin production at the mid- to late logarithmic growth phase. Previous studies in our laboratory showed that the mid- to late logarithmic phase of growth is the point of maximal AH production (1, 21). The amount of AH produced by these isolates ranged from 40 to 160 HU_{50}/ml. There was no correlation between the amount of hemolysin produced by the isolates and the anti-AH antibody titer in the homologous patient serum (data not shown).

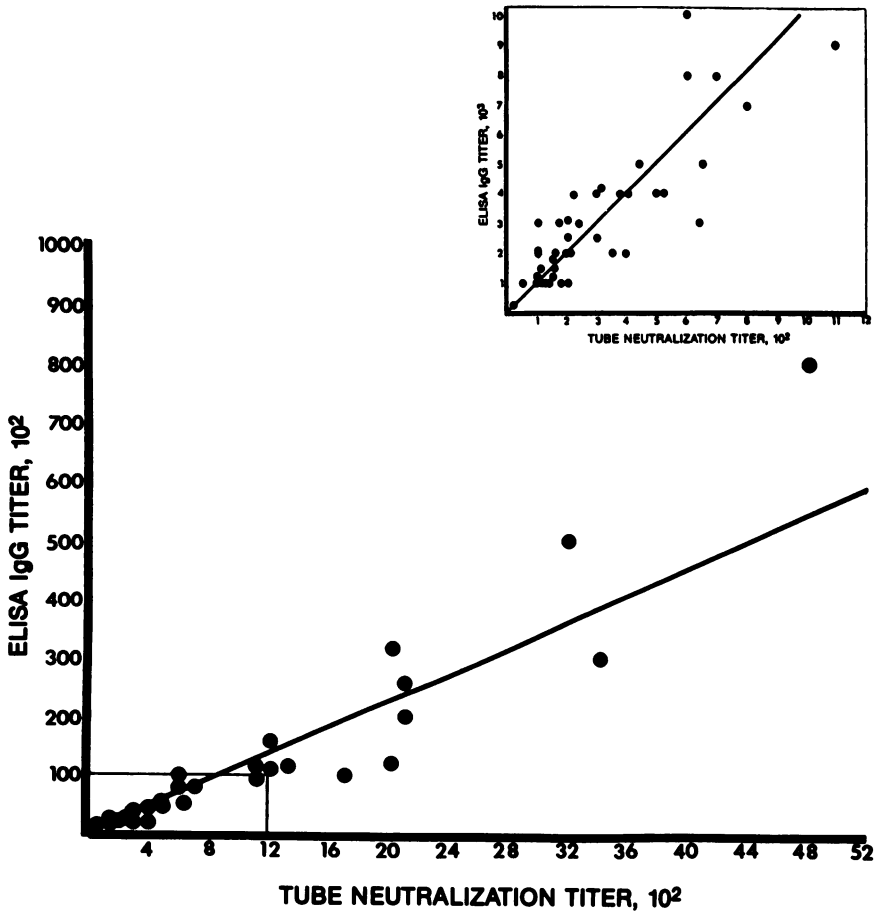

FIG. 2. Correlation between TN assay and ELISA in the measurement of AH antibody. Inset shows correlation at low anti-AH titer levels.

TABLE 1. *E. coli* AH antibody in patient sera before and after adsorption with LPS

Patient no.	Titer[a]											
	Anti-AH						Anti-LPS					
	Adsorbed sera[b]			Unadsorbed sera[c]			Adsorbed sera[b]			Unadsorbed sera[c]		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
44	12,800	<100	100	12,800	<100	300	<100	<100	<100	200	<100	100
47	800	<100	<100	1,600	<100	<100	<100	<100	<100	400	<100	<100
48	400	<100	<100	700	<100	<100	<100	<100	<100	200	<100	<100
50	800	<100	<100	1,000	<100	<100	<100	<100	<100	400	100	<100
53	100	<100	<100	400	<100	<100	<100	<100	<100	1,000	<100	<100
35	800	100	<100	2,000	<100	<100	<100	<100	<100	3,200	200	200
37	800	100	<100	1,000	<100	100	<100	<100	<100	2,000	100	200

[a] Serum titers were determined by ELISA.
[b] LPS antibody adsorbed by incubating sera with 250-μg/ml dose of H79 LPS at 37°C for 1 h followed by 4°C overnight.
[c] Sera incubated with 0.9% saline.

AHs produced by these four isolates were neutralized equally by a mouse monoclonal neutralizing antibody (D12E4E12, IgG2a) (Table 3) in a TN assay. Table 3 also shows TN assay results with several patient sera. Each serum gave identical titers with hemolysin from all four AH isolates. The sera reacted identically with AH isolates irrespective of the patient source.

Analysis of AH proteins by SDS-polyacrylamide gel electrophoresis. The electrophoretic protein profiles of AH detected by Coomassie blue staining were compared with those identified by monoclonal antibody probes and patient serum containing antibodies against AH (Fig. 3). The AH protein profile, as identified by monoclonal antibody (lane C) and by sera from infected patients with AH antibody only (LPS-adsorbed serum) (lane D), is similar to the Coomassie blue profile (lane B). The 107,000-dalton hemolysin protein was identified in each case. The stepladder pattern of LPS (unadsorbed sera, lane E) was lost when serum from an infected patient was adsorbed with H79 LPS (lane D). As expected, serum from a control patient without a hemolytic *E. coli* infection (titer, <100 by TN assay) did not react with AH protein (lane F).

Some sera had a high anti-AH titer but a low anti-LPS titer. Serum with an H79 LPS antibody titer of less than 100 was used as a probe of AH proteins without adsorption with H79 LPS. This was compared with the profile obtained with serum containing a high titer (1,000) of anti-LPS antibody (Fig. 4). The 107,000-dalton AH protein and several lower-molecular-weight bands were recognized by both sera (lanes B and C). The stepladder band pattern of LPS seen with high-titer anti-LPS antibody serum (lane C) was absent when LPS was probed with the low-titer serum (lane B).

Clinical correlation. The infection group showed high anti-AH titers (mean: TN, 940; ELISA, 8,450), whereas the control group showed low antibody titers (mean: TN, 123; ELISA 1,520) (Fig. 5). The cluster diagram highlights the broad titer range of patients with UTI included in the infection group. Antibody titers varied from a low of <100 to a high of 13,000 by the TN assay (<1,000 to 130,000 by ELISA). Six immunocompromised patients were in the lower titer range, while the three patients with disseminated infection were in the upper titer range. Comparison of the two population groups by the Mann-Whitney U test showed that the groups were significantly different ($P < 0.01$).

AH-neutralizing antibody was measured during the course of infection in three patients infected with hemolytic *E. coli* (Fig. 6). Patient A showed rising bacterial counts in urine till day 11 of infection. She was treated with antibiotics and on discharge had no bacteria in the urine. Anti-AH titers showed a rise during the period of infection and peaked on day 20 (titer of 6,000). This was followed by a sharp fall to a titer of 3,800 on day 27 after successful treatment with antibiotics. Patients B and C had acute cystitis and showed a minimal increase in antibody during the period of infection. In patient B, the bacterial count in urine peaked on day 11, and on discharge he still had 10^4 bacteria per ml in the urine. The anti-AH titer remained steady at 380 for the first 15 days followed by a gradual increase. The titer peaked on day 30 of

TABLE 2. AH-neutralizing antibody in patient sera after adsorption of LPS antibody

Patient no.	Anti-AH titer[a]	
	Adsorbed[b]	Unadsorbed[c]
44	12,800	13,000
47	2,400	2,400
48	1,600	1,500
50	1,600	1,600
53	3,200	3,200
35	2,700	2,500
37	1,600	1,600

[a] Serum titers were determined by the TN assay.
[b] LPS antibody was adsorbed by incubating sera with 250-μg/ml dose of H79 LPS at 37°C for 1 h followed by 4°C overnight.
[c] Sera were incubated with 0.9% saline instead of LPS.

TABLE 3. TN assay with AH from various *E. coli* isolates

AH supernatants from culture no.:	Titer[a]				
	Serum 23	Serum 37	Serum 43	Serum 58	Monoclonal antibody[b]
23	1,600	570	1,600	400	1,900
32	1,400	500	1,500	370	1,900
37	1,600	600	1,600	400	2,100
43	1,800	580	1,800	380	2,100
H79[c]	1,500	600	1,600	370	2,600

[a] Titration by standard TN assay.
[b] D12E4E12, a mouse monoclonal neutralizing antibody, was used.
[c] AH from *E. coli* H79 as a control.

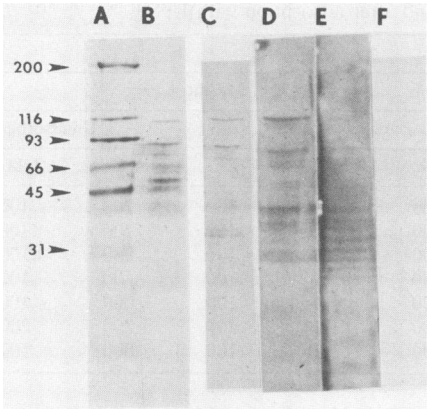

FIG. 3. Comparison of reactivity of AH proteins with hemolysis-neutralizing monoclonal antibodies and antibodies from patient sera. Lanes A and B, Coomassie blue-stained SDS-polyacrylamide gel electrophoresis profiles: A, protein standards with molecular sizes expressed as kilodaltons; B, AH (1,000 HU_{50}). Lanes C to F, Immunoblots of AH proteins on nitrocellulose: C, D12E4E12 ascites probe; D, serum (adsorbed with H79 LPS) from infected patient as probe; E, serum (unadsorbed) from infected patient as probe; F, serum from patient without hemolytic *E. coli* infection (titer, <100) as probe.

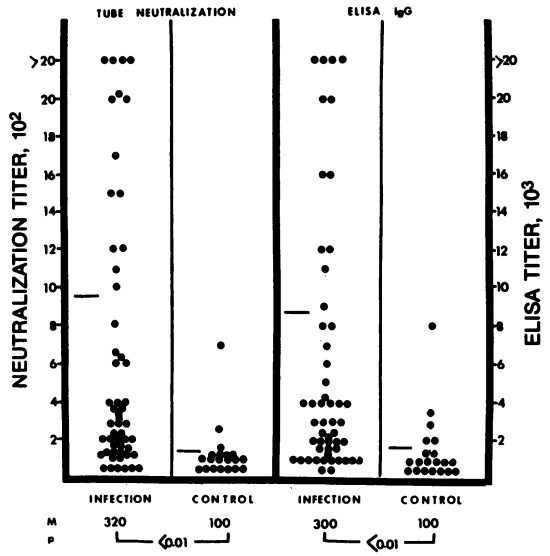

FIG. 5. Correlation of AH antibody and immunoglobulin levels with disease. The population in the study was divided into infection and control groups. The line refers to the calculated mean. M, Median. *P* value was determined by Mann-Whitney U test.

infection (800). On discharge, he still had an antibody titer of 500. Patient C showed a minimal rise in antibody titer, peaking at 400 on day 10. She was treated with antibiotics, and on day 14 of infection no growth was seen in the urine culture.

DISCUSSION

We measured the antibody response of humans to *E. coli* AH by neutralization tests and ELISA. The presence of anti-AH antibody in infected patients confirms the fact that AH is produced by the bacteria in the host. The use of monoclonal antibodies and patient sera in serological assays and as probes for hemolysin in SDS-polyacrylamide gel electrophoresis studies confirms earlier reports using an antibody to an impure preparation of hemolysin that showed that AHs from different *E. coli* isolates are antigenically identical (7, 14, 16, 20). These results also support the findings of Hacker and Hughes (12), who showed that AH determinants of both plasmids and chromosomes code for an identical or similar protein(s). No correlation was shown between the amount of AH produced in vitro and the antibody response in humans. This suggests that in vivo

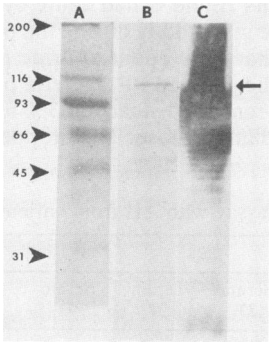

FIG. 4. Comparison of reactivity of AH proteins with hemolysin-neutralizing antibodies from patient sera. Lane A, Coomassie blue stain of protein standards with molecular sizes expressed as kilodaltons. Lanes B and C, immunoblots of AH proteins on nitrocellulose: B, serum from infected patient (with anti-LPS antibody titer <100 by ELISA) as probe; C, serum from infected patient (with anti-LPS antibody titer of 1,000 by ELISA) as probe. Arrow indicates the 107,000-dalton hemolysin protein.

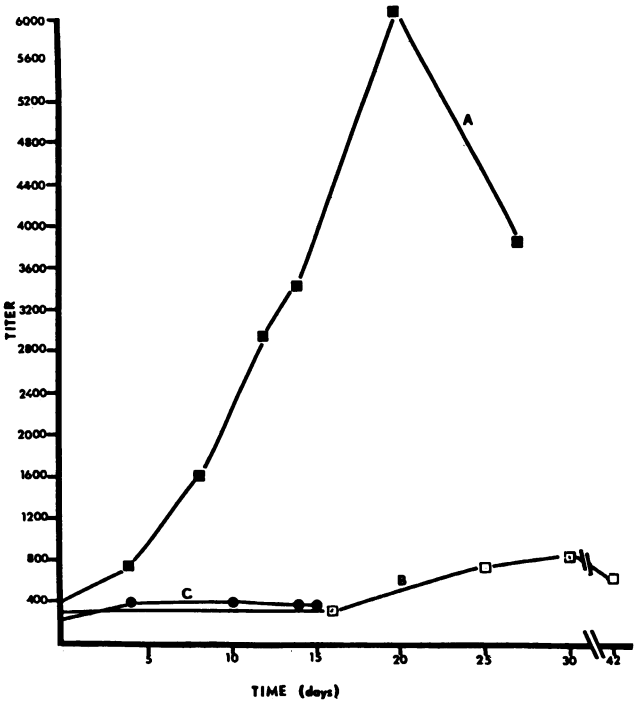

FIG. 6. *E. coli* AH-neutralizing antibody in serial samples of patient sera. Symbols: ■, patient A, 84-year-old woman with *E. coli* UTI and septicemia; □, patient B, 89-year-old man with acute cystitis; ●, patient C, 69-year-old woman with cystitis.

production of AH may not parallel in vitro production or that invasiveness, severity, or length of the infection may be more important than the amount of AH produced. Our data and that of others (7, 16, 20) suggest that severity of infection correlates with antibody titer. Further, we showed that LPS complexed with AH (1) does not interfere with measurement of anti-AH.

The three immunoassay techniques compared included a TN assay, an MN assay, and an ELISA. Of the three assays, the TN and MN assays were equally sensitive when compared to each other. The ELISA appears to be the most sensitive assay. However, the ELISA measures antibody to total AH (active and inactive) as well as other components in the crude AH preparation, thereby decreasing the specificity of the assay. The TN and MN assays are specific and measure only antibody that reacts with AH. As a quick, sensitive, and AH-specific laboratory assay, the MN assay appears the best. With this assay, a large number of serum samples can be run at one time, only small amounts of sera are needed, the incubation time is only 2 h, and the results can be read easily and quickly.

The major immunoglobulin class associated with AH-neutralizing antibody was IgG. Low levels of IgM and IgA were found in a few sera. Most of the anti-LPS antibody found in patient sera was of the IgG class. The low IgM titers are surprising because an acute immunological response to LPS is usually a T-cell-independent IgM response. However, the LPS antibody response can be variable, with production of IgM, IgG, and IgA (22). Previous studies done in our laboratory indicate that AH in *E. coli* culture supernatants is complexed with LPS (1). H79 LPS adsorbed the LPS antibody without altering anti-AH levels, indicating that AH and LPS, though complexed, are antigenically different. This is confirmed by the presence of AH protein bands and by the absence of LPS bands on immunoblotting with adsorbed serum.

Our study showed that patients infected with *E. coli* have high neutralizing antibody titers against AH. Like others, we also showed that AH titers increased during the course of illness (16, 20) and correlated with the invasiveness and severity of the infection (7). It appears that immunocompromised patients have low AH antibody titers, while patients with disseminated *E. coli* infections have high anti-AH titers. The low anti-AH titers in our control group support the findings of Hugo et al. (14) and indicate past infection with hemolytic *E. coli*. Sixty percent of the control population were females, who are prone to asymptomatic bacteriuria and UTI.

Serial antibody titer studies on three patients showed variation in antibody levels based on severity and duration of infection, effectiveness of treatment, and individual patient response. A patient with a disseminated *E. coli* septicemia showed a fourfold increase in titer followed by a drop, probably in response to antibiotic therapy. Two patients with a mild cystitis showed a minimal rise in titer. One was successfully treated, and antibody titers returned to base line. A continuing infection in the other patient, as evidenced by bacterial counts in urine, was reflected by an ongoing antibody response.

The data suggest that antibody response could be used to monitor infections caused by hemolytic *E. coli*. This could be of value in those situations in which isolation of an organism is difficult, in which its presence is of questionable significance, or in retrospective studies. An important question to be answered is the significance of the antibody response to the hemolysin in disease.

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