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Protective Capacity of Antibodies Against *Escherichia coli* O and K Antigens

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Antibodies to *Escherichia coli* O and K antigens were raised in rabbits by repeated immunizations with whole, Formalin-killed and, later, live bacteria. The serum antibody levels were determined with the ammonium sulfate precipitation technique after radioiodinating the antigens. The K antigens had to be conjugated to proteins before labeling. Such conjugations were performed using cyanogen bromide for the K1 antigen and bisdiazobenzidine for the K13 antigen. The protective capacities of the rabbit antisera were tested in intraperitoneally infected mice. The protective capacity of the antisera was expressed per ammonium sulfate precipitation titer. The results showed a significantly higher protective effect for the antibodies against the K1 and K13 antigens than for the antibodies against the O2 and O6 lipopolysaccharides.

The cell wall in *Escherichia coli* bacteria is a complex structure containing different virulence factors and antigens. The lipopolysaccharide (LPS) O antigen and the polysaccharide capsular K antigen have been considered the most important of such structures (10, 13, 22, 23, 27, 28). These antigens occur in many serologically different variations (18).

After exposure to *E. coli* bacteria, an individual in most cases forms antibodies against the O antigen (2, 3, 6, 12, 20, 29), whereas antibodies against the K antigen occur less frequently (16). Such anti-O and -K antibodies are protective in experimental infections such as peritonitis in mice and pyelonephritis in rabbits (1, 15, 17, 19, 26, 31). Furthermore, they have been found protective in cases with bacteremia caused by gram-negative bacteria (32). However, there is no study evaluating the relative protective efficiency of the anti-O and anti-K antibodies. The purpose of the present investigation was to compare the protective capacity of rabbit anti-*E. coli* O and K antibodies in mice intraperitoneally infected with *E. coli*.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains were obtained from the WHO Escherichia Center, Statens Seruminstitut, Copenhagen, Denmark. The bacteria used for immunizations were *E. coli* strains O2:K2ab:H1, O6:K13:H1, O2:K1:H4, and O22:K13:H1 with the WHO designations Su 1242, Su 4344/41, U9/41, and E14a. In the protection experiments the mice were challenged with *E. coli* O2:K1:H4, O6:K2a2c:H1, O1:K1:H7, and O6:K13:H1 with the WHO designations U9/41, Bi 7458/41, U5/41, and Su 4344/41 to determine the separate protective effect of anti-O and anti-K antibodies.

Determination of 75% lethal doses (LD_{75}) for the bacterial strains was done according to Reed and Muench (24). LD_{75} values were 5×10^7 for *E. coli* O6:K2a2c:H1 and 5×10^6 for O6:K13:H1, O2:K1:H4, and O1:K1:H7.

Antigens. In the ammonium sulfate precipitation (ASP) technique, LPS antigens of the O2:K1:H4 and O6:K2a2c:H1 *E. coli* serotypes were extracted with phenol-water (23). K1 and K13 antigens were isolated by using Cetavlon precipitation from the O1:K1:H7 and O22:K13:H1 strains.

The isolated LPS preparations contained no protein, as measured by the Lowry technique, corresponding to less than 1 mg of protein per 100 mg of material (21). They contained no K polysaccharide as tested by immunodiffusion as well as inhibition experiments using indirect hemagglutination. These analyses thus showed less than 1 mg of K antigen per 100 mg of the O antigen preparation. A contamination of K antigen in the LPS should, however, not influence the result, since the method for labeling LPS did not work for K polysaccharide.

The purified K antigen was produced from bacteria grown in 1 liter of tryptic soy broth in a 2-liter bottle at 37°C for 5 h. After 3.5 h, 0.5 g of glucose was added. The pH was continuously adjusted to 7.4 using 2.5 mol of NaOH per liter. The culture was centrifuged, and the supernatant was diluted to 3 liters using distilled water, whereupon 1 g of cetylmethylammonium bromide (Cetavlon) was added. This solution was then stirred at 37°C for 1 h.

After centrifugation ($3,000 \times g$) for 30 min, the sediment was dissolved in distilled water and stirred overnight at 4°C. Then 55.5 g (0.5 mol) of $CaCl_2$ was added, and the solution was again stirred for 15 min.

Ethanol was then added to a final concentration of 20%. After centrifugation at $3,000 \times g$ for 30 min,

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the supernatant was taken and ethanol was added to a final concentration of 80%. After centrifugation for 3,000 × *g* for 30 min, the sediment was dissolved in 500 ml of saturated potassium acetate, pH 7.4, and 0.5 kg of phenol was added.

After stirring for 20 min, the phased solution was centrifuged for 1 h at 3,000 × *g*. Finally, the upper phase was taken as K antigen. This was lyophilized and stored at 4°C until used.

The isolated K antigen contained no protein, as determined by the Lowry technique (21), corresponding to less than 1 mg of protein per 100 mg of material. There was no contamination of O LPS as tested by immunodiffusion. Furthermore, no O antibodies were seen in rabbits immunized with isolated K antigen.

The K1 antigen was conjugated to bovine serum albumin using the method described by Axén and co-workers (5). Thus, 10 mg of purified K1 substance was dissolved in 0.5 ml of distilled water. A solution of 0.005 mg of cyanogen bromide (Merck, Darmstadt, Germany) in 0.2 ml of distilled water was added, and the pH was raised to 10.5 by adding 1 mol of NaOH per liter. The pH was kept at 10.5 for 30 min by continued addition of a total of 0.25 ml of 1 mol of NaOH per liter. The cyanogen bromide-activated K1 antigen was desalted on a microcolumn of Sephadex G-25 (Pharmacia, Uppsala, Sweden) and eluted with 0.005 mol of $NaHCO_3$ per liter. The void volume fractions were then pooled and lyophilized. The material was dissolved in 0.1 mol of $NaHCO_3$ per liter, and 1.5 mg of bovine serum albumin was added. The mixture was incubated at room temperature for 16 h during vertical rotation. Thereafter, the sample was dialyzed against 2 × 1 liter of ethanolamine buffer, 0.05 mol/liter (Merck, Darmstadt), in 0.1 mol of $NaHCO_3$ per liter for 24 h. The sample was then gel filtered on a microcolumn of Sephadex G-25, and elution was performed with 1.0 mol of NaAc-HAc buffer (pH 4.0) per liter. The eluted fractions were tested with double diffusion in gel (30), using a precipitating anti-K1 antiserum, and the antigen-containing void volume fractions were pooled and lyophilized until used.

The K13 antigen was not stable at the high pH used for cyanogen bromide activation and did not contain any immunoprecipitating material after such treatment. This antigen was instead conjugated to bovine serum albumin in 0.05 mol of phosphate buffer (pH 7.4) per liter, using bisdiazobenzidine (Merck, Darmstadt), according to Andersson (4). A 0.5-ml amount of 5 mg of K13 antigen and 0.5 mg of bovine serum albumin, respectively, was mixed with 0.05 ml of a fresh solution of bisdiazobenzidine diluted 1/16. The mixture was incubated for 20 min at room temperature in the dark. This solution was desalted by gel filtration on a microcolumn of Sephadex G-25. The fractions were tested using double diffusion in gel (30). The antigen-containing void volume fractions were pooled and lyophilized.

Radioiodination of antigens. The chloramine T method originally described by Hunter and Greenwood was used (11). Thus, 10 mg of antigen in 100 μl was labeled with 4 mCi of [^{125}I]Na (AB Atomenergi, Studsvik, Sweden) for 1 min, using 25 μl of 0.05-mol/liter phosphate buffer (pH 7.4) containing 8 mg of chloramine T per ml. The reaction was stopped by the addition of 100 μl of sodium metabisulfite (4.8 mg/liter). After the addition of 100 μl of potassium iodide (20 mg/ml), the solution was dialyzed against 2 liters of phosphate buffer, pH 7.2 (0.05 mol/liter).

ASP technique for antibody determination. The ASP technique originally described by Farr (7) was used as described previously (2). Thus, about 5 μg of antigen in 0.2 ml of borate buffer (pH 8.2) was added to 0.2 ml of threefold dilutions of antiserum. The antigen-antibody complexes formed were precipitated with 0.4 ml of saturated ammonium sulfate. The highest serum dilution precipitating 20% of the antigen added was recorded as the titer. To ascertain a similar antibody-binding capacity of the antigen preparations in the antibody titrations with the ASP technique, the different antigens employed were used in concentrations showing equal immunoprecipitating capacities. This was performed by double diffusion in gel according to Wadsworth (30). For this purpose two antisera with similar precipitating capacities of purified but unconjugated antigens were used. The antibody-precipitating capacity was then analyzed by twofold serial dilutions of the prepared antigen.

Immunizations. Antiserum to the bacterial strains was produced in rabbits as earlier described (10). The rabbits were given six weekly intravenous injections, the first three with Formalin-killed bacteria and the last three with live bacteria. The amount of bacteria in the solutions used for injection was 10^8/ml. The volumes given were as follows: 0.25, 0.25, 0.50, 0.50, 1.0, and 1.0 ml. All antisera were checked for the presence of O antibodies as well as K antibodies before use.

Protection experiments. The antisera were tested by passive immunization in intraperitoneally infected mice as described before (15). The bacterial concentration giving 1 LD_{75} was established for each bacterial strain. Groups of eight mice were given intraperitoneally 0.5 ml of antiserum diluted 1/16 or higher, and 4 h later they were challenged with 1 LD_{75} of the bacteria. The number of surviving animals was preliminarily recorded after 24 h and finally after 48 h. A serum dilution causing protection of six out of eight mice was recorded as the protection titer. As a control in each experiment, a group of eight animals was injected with saline instead of antiserum. Furthermore, control experiments using preimmune serum instead of saline were also done, showing similar results. Of these animals, two should survive 48 h after challenge with 1 LD_{75}; otherwise the experiment would be repeated. The somewhat different virulence of the various strains did not seem to affect the results, as judged from very similar slopes of titration curves of the LD.

Control experiments had been performed in a pilot study to check the validity of the test system and to ascertain that no other antigens of, for instance, a protein nature significantly influenced the test system. Thus, antisera raised against the *E. coli* O2:K2ab:H1 did not protect against challenge with *E. coli* O6:K13:H1 or O1:K1:H7; antisera raised against the *E. coli* O6:K13:H1 did not protect against *E. coli* O2:K1:H4 or O1:K1:H7; antisera raised

against *E. coli* O2:K1:H4 did not protect against *E. coli* O6:K2a2c:H1 or O6:K13:H1; antisera raised against *E. coli* O22:K13:H1 did not protect against *E. coli* O2:K1:H4, O6:K2a2c:H1, or O1:K1:H7.

RESULTS

All serum samples included in the study contained varying levels of antibodies to the O and K antigens used for immunization. The amounts of anti-K1 antibody were lower than those of anti-K13 as well as anti-O2 and -O6 antibodies. The highest antibody titers were recorded in the antisera raised against the *E. coli* O2 and the K13 antigens (Table 1).

In the protective system utilizing intraperitoneally infected mice, the antisera against K13 antigen mediated the best protection. This effect was somewhat better than that for antibodies against K1 and O2 antigens (Table 1). The antisera raised against the O6 antigen showed a much weaker protective effect than the other antisera to their respective serotypes.

The protective capacity of the antisera, expressed as "protective titer/ASP titer" (1), was calculated. Such an evaluation of the protective capacity per antibody unit showed that the antisera to the K1 and K13 antigens were much more efficient in their protective function than those against the O2 and, in particular, those against the O6 antigen (Table 1).

DISCUSSION

The present results corroborate previous studies showing an immunologically specific protective effect of antibodies raised against the *E. coli* O as well as against capsular K antigens (1, 13, 16). Furthermore, the present investigation extends previous studies by showing a higher protective capacity of the antibodies against the capsular K1 and K13 antigens compared to those against the O2 and O6 antigens in intraperitoneally infected mice. These results could be of significance for future immunization experiments in humans to obtain protection against infection, since only a limited number of different K antigens are found in the majority of *E. coli* bacteria causing urinary tract infection (14), whereas a great number of different O serotypes can be found (20).

It is tempting to speculate about the marked protective effect of the antibodies against the K antigens. Thus, these antigens are of biological significance because of their apparent function to increase the capacity of the bacteria to penetrate into parenchymal tissues (8, 9, 13). Accordingly, the K1 antigen is especially frequent in *E. coli* strains causing pyelonephritis (13, 14) as well as neonatal meningitis (25). The low antibody levels to the K1 structure correspond to its poor immunogenicity, as noticed earlier (16).

The relatively low protective capacity of the anti-O antibodies is well in accordance with clinical observations. Apparently patients can attract pyelonephritis caused by *E. coli* in spite of having high serum antibody titers to their particular O antigen (3, 29). So far no corresponding clinical investigation concerning K antibodies in patients has been performed.

The present investigation concerning serum antibodies indicates that K antibodies in particular may be significant for protection against gram-negative bacterial infections, including urinary tract infections. However, preliminary

TABLE 1. *Antibody titers in rabbit sera against the E. coli O2, O6, K1, and K13 antigens determined with the ASP technique: protective capacity in peritoneally infected mice as well as protective capacity in relation to the ASP titer*

Serum no.	Anti-O2			Anti-O6			Anti-K1			Anti-K13		
	ASP titer	Protection titer	Protection titer/ ASP titer[b]	ASP titer	Protection titer	Protection titer/ ASP titer[b]	ASP titer	Protection titer	Protection titer/ ASP titer[c]	ASP titer	Protection titer	Protection titer/ASP titer
1	26	<16	<0.62	26	<16	<0.62	6	<16	<2.67	48	<16	<0.33
2	63	32	0.51	29	<16	<0.55	6	64	10.67	84	128	1.52
3	78	32	0.41	29	<16	<0.55	8	128	16.00	81	256	3.16
4	26	16	0.62	12	<16	<1.33	8	64	8.00	108	>2,048	>18.96
5	81	128	1.58	25	<32	<1.28	5	64	12.80	26	<16	<0.62
6	78	64	0.82	27	<16	<0.59	3	32	10.67	63	>1,024	>16.25
7	75	256	3.41	36	<16	<0.44	4	32	8.00	63	>512	>8.13
8	87	256	2.94	18	<16	<0.88	8	16	2.00	60	>512	>8.53
9	84	128	1.53	54	<32	<0.59	8	<16	<2.00	69	128	1.86
10				51	<16	<0.31	4	64	16.00			
11				45	<16	<0.36	6	64	10.67			
12							4	64	16.00			

[a] Significantly different from O6 ($P < 0.005$), K1 ($P < 0.005$), K13 ($P < 0.05$).
[b] Significantly different from K1 ($P < 0.01$), K13 ($P < 0.005$).
[c] Not significantly different from K13 ($0.05 < P < 0.10$).

data also suggest that locally formed antibodies may contribute to the protection (B. Kaijser et al., manuscript in preparation). Thus, comparisons between serum and locally formed antibodies of varying specificities should be performed in a way comparable to that described in this communication. This would ascertain the origin and specificity of the antibodies that are most efficient in affording protection against gram-negative bacterial infections.

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