Opsonizing and Bactericidal Activity of Antibodies Against Common Antigen of *Enterobacteriaceae*

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ABSTRACT

DOMINGUE, GERALD J. (State University of New York at Buffalo, Buffalo, N.Y.), AND ERWIN NETER. Opsonizing and bactericidal activity of antibodies against common antigen of *Enterobacteriaceae*. J. Bacteriol. 91:129-133. 1966.—In addition to the well-known O, H, K, and Vi antigens, *Enterobacteriaceae* produce a common antigen, which was first identified by hemagglutination tests with *Escherichia coli* O14 antiserum. Studies on the biological significance of this antigen by in vitro phagocytic experiments have revealed that opsonization is markedly enhanced in the presence of the corresponding antibody, rabbit polymorphonuclear leukocytes, and normal rabbit serum. This effect is specific, because *Pseudomonas aeruginosa*, which is devoid of this antigen, is not opsonized under these conditions, and removal of the antibody by absorption markedly reduces the uptake of enteric bacteria containing the antigen. Opsonization thus represents another method for the study of this antigen-antibody system. Bactericidal tests have revealed that antibodies against this antigen, engendered in rabbits by different strains of enteric bacteria and various procedures, are bactericidal for *E. coli* O14 but not for other enteric bacteria, possibly due to previously demonstrated differences between the antigen moieties obtained from these microorganisms.

The common antigen (CA) of *Enterobacteriaceae*, first described in 1962 by Kunin et al. (10), probably had escaped detection for many years, because it can be demonstrated only by means of the hemagglutination and hemolysis tests and by neither bacterial agglutination nor precipitation procedures. In addition, intravenous injection of enteric bacteria other than *Escherichia coli* O14 does not result in formation of antibodies against this antigen. Subsequent studies have revealed, however, that such antibodies are readily engendered, if crude CA is injected into the footpad of rabbits together with Freund's adjuvant, or in the cell-attached rather than the soluble state (7, 12, 19). More recently, it has been shown that CA can be separated from the O antigen by means of 85% ethyl alcohol, the former being soluble and the latter insoluble (16). Surprisingly, the ethyl alcohol-soluble fraction proved to be highly immunogenic upon intravenous injection in the rabbit. Further investigations have revealed that the O antigen (lipopoly-

saccharide or its lipoid A component), when injected together with CA, interferes with the antibody formation against the latter (18 and Whang et al., unpublished data).

At the present time, only limited information is available on the biological significance of this antigen-antibody system. Antibodies against CA are present in the serum of healthy subjects and in commercial γ -globulin preparations, and it was shown that some children with enteric bacterial infection develop such antibodies, although in low titers (20). Suzuki et al. (17) observed that hemolysis takes place in immunized, but not in nonimmune, rabbits upon the injection of autologous erythrocytes modified with CA.

The present experiments were undertaken to determine whether antibodies against CA enhance phagocytosis of enteric bacteria and to extend the observations of Kunin and Beard (9) on the bactericidal effects of these antibodies.

MATERIALS AND METHODS

Cultures. Strains of *E. coli* O14, *E. coli* O111, *E. coli* O55, *E. coli* K-12, *Salmonella typhimurium*, and *S. choleraesuis* were grown in Brain Heart Infusion at

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37 C for either 4 or 18 hr. Colonies were taken from blood-agar for seeding purposes.

Rabbit polymorphonuclear leukocytes. The method of Cohn and Morse (5) was employed for producing and harvesting homogenous populations of polymorphonuclear leukocytes from peritoneal exudates of albino rabbits. The leukocytes and bacteria were suspended in Hanks' bovine albumin glucose solution (HBG), prepared according to the method of Martin and Green (11).

Normal sera. Normal rabbit serum was obtained from rabbits kept especially for this purpose. The fetal calf serum was purchased from Grand Island Biological Co., Grand Island, N.Y. The piglet serum was kindly supplied by Dennis W. Watson, University of Minnesota. The sera were stored in small volumes at -20 C for no longer than 30 days and were used in a final concentration of 10% in both phagocytic and bactericidal experiments.

Antisera. Antisera against CA were prepared by several methods. Rabbits were injected intravenously with either heat-killed suspensions of *E. coli* O14 or with the ethyl alcohol-soluble fraction of supernatant liquids obtained from *S. typhimurium* (16); supernatant liquid from *S. typhimurium* together with complete Freund's adjuvant was injected into the footpad of rabbits (7); additional rabbits were injected once with killed *S. typhosa* or supernatant liquid of *E. coli* O111 suspension for priming purposes, followed by a single intravenous injection of 1 ml of ethyl alcohol-soluble fraction of *S. typhimurium* (unpublished data).

Absorption of antisera. Sediment of CA-modified erythrocytes was mixed with CA antisera which had been inactivated at 56 C for 30 min, and absorption was allowed to proceed for 1 hr in a cold room (21).

Phagocytosis. Each mixture for phagocytosis contained 1.6 ml of leukocyte suspension (30×10^6 per milliliter), 0.2 ml of bacterial suspension (960×10^6 per milliliter, resulting in a multiplicity of four bac-

teria to one leukocyte), and 0.2 ml of normal, unheated serum (rabbit, fetal calf, or piglet) or 0.2 ml of antiserum appropriately diluted in normal serum. Approximately 99% of the leukocytes and bacteria were viable, as determined by the trypan blue dye exclusion test for leukocytes and by plate counts for bacteria. The tubes were stoppered with sterile rubber stoppers and rotated, end-over-end, at 37 C at 8 rev/min on a roller-drum type rotator (New Brunswick Scientific Co., New Brunswick, N.J.).

Films, prepared at various times, were stained with methylene blue; 500 leukocytes were counted, and the number of leukocytes containing ingested bacteria was noted.

Serum bactericidal test. Infusion broth cultures were washed twice in HBG and diluted to yield approximately 7.6×10^4 to 1.4×10^6 bacteria per milliliter. For the tests, each tube contained 0.2 ml of fresh guinea pig complement in a final dilution of 1:100, 1.4 ml of HBG, 0.2 ml of normal rabbit serum or appropriately diluted antiserum, and 0.2 ml of bacteria suspended in HBG.

The tubes were placed in a water bath at 37 C. At different time intervals, 0.1 ml was removed and diluted in physiological saline; then, 1 ml of each dilution was mixed with 15 ml of melted brain-veal-agar. Accurate counts from 10^1 to 10^6 bacteria per milliliter were made possible.

RESULTS

In the first series of experiments, the effects of antibodies against the CA on opsonization of enteric bacteria were investigated. Representative results obtained with antisera produced by different methods on two test organisms are shown in Table 1. It is evident that CA antibodies opsonize both *E. coli* O14 and *S. typhimurium* for phagocytosis by polymorphonuclear cells, when com-

TABLE 1. Effect of antibodies against common antigen of enteric bacteria on phagocytosis by polymorphonuclear leukocytes

Antiserum* (1:100)	Test organism	Percentage of leukocytes containing ingested bacteria at			
		0 min	10 min	20 min	30 min
<i>Salmonella typhimurium</i> with Freund's adjuvant	<i>Escherichia coli</i> O14	10	36	58	54
<i>S. typhimurium</i> (ethyl alcohol-soluble fraction)	<i>E. coli</i> O14	17	52	78	70
<i>E. coli</i> O14	<i>E. coli</i> O14	36	80	95	80
Normal rabbit serum	<i>E. coli</i> O14	8	20	23	33
<i>S. typhimurium</i> (ethyl alcohol-soluble fraction)	<i>E. coli</i> K-12	9	67	75	89
Normal rabbit serum	<i>E. coli</i> K-12	9	39	43	40
<i>E. coli</i> O14	<i>S. typhimurium</i>	18	69	80	71
Normal rabbit serum	<i>S. typhimurium</i>	20	28	32	22

* Antisera containing antibodies against common antigen engendered by various procedures (see text).

TABLE 2. *Effects of antibodies against common antigen on phagocytosis of Pseudomonas aeruginosa and of antibody absorption on that of Escherichia coli O14*

Antiserum* (1:100)	Test organism	Percentage of leukocytes containing ingested bacteria at			
		0 min	10 min	20 min	30 min
<i>Salmonella typhimurium</i> (ethyl alcohol-soluble fraction)	<i>P. aeruginosa</i>	0.8	5	11	12
Normal rabbit serum	<i>P. aeruginosa</i>	0.4	11	13	23
<i>S. typhimurium</i> (ethyl alcohol-soluble fraction) absorbed†	<i>E. coli</i> O14	20	18	29	24
<i>S. typhimurium</i> (ethyl alcohol-soluble fraction)	<i>E. coli</i> O14	8	88	93	89
Normal rabbit serum	<i>E. coli</i> O14	24	32	34	19

* Antibodies against common antigen engendered with ethyl alcohol-soluble fraction of *S. typhimurium*.

† Absorption with CA-modified erythrocytes resulted in 98% reduction of hemagglutinin titer.

TABLE 3. *Bactericidal effects of antibodies against common antigen and complement on enteric bacteria*

Antiserum* (1:100)	Test organism	Number of viable bacteria/ml at				Per cent killed at		
		0 min	15 min	30 min	60 min	15 min	30 min	60 min
<i>Salmonella typhosa</i>	<i>Escherichia coli</i> O14	7.2×10^4	2.8×10^4	$<10^3$	$<10^3$	61	>99	>99
<i>E. coli</i> O111	<i>E. coli</i> O14	7.4×10^4	1.0×10^4	$<10^3$	$<10^3$	87	>99	>99
<i>E. coli</i> O14	<i>E. coli</i> O14	7.0×10^4	1.0×10^4	$<10^3$	$<10^3$	85	>99	>99
<i>S. typhimurium</i>	<i>E. coli</i> O14	8.2×10^4	5.0×10^4	1.0×10^5	$<10^3$	40	>99	>99
Normal rabbit serum	<i>E. coli</i> O14	6.6×10^4	7.4×10^4	1.10×10^5	7.5×10^4	G†	G	G
<i>E. coli</i> O14	<i>S. typhimurium</i>	3.4×10^4	1.3×10^5	1.15×10^5	1.2×10^5	G	G	G
Normal rabbit serum	<i>S. typhimurium</i>	3.8×10^4	7.0×10^4	1.13×10^5	1.56×10^5	G	G	G

* Antisera containing antibodies against common antigen engendered by various procedures (see text).

† G = growth.

parison is made between degree of phagocytosis in the presence of immune and normal sera in identical dilution. Attention may be called to the fact that, with the exception of *E. coli* O14 antiserum, all antisera were prepared by immunization with enteric bacteria unrelated in their O antigens to the test organisms. Essentially identical results were obtained with *E. coli* K-12. Titration of the CA antiserum revealed that at a dilution of 1:100 it was most effective; at a dilution of 1:1,000 it showed slight opsonization, and at a dilution of 1:10,000 it was ineffective.

To prove that opsonization by CA antiserum is specific, two series of experiments were carried out (Table 2). In one, *Pseudomonas aeruginosa*, which does not contain CA, was used as test organism, and in the other, CA antiserum was used

before and after absorption of CA antibodies. It is clear that CA antibodies do not enhance phagocytosis of *P. aeruginosa*, when comparison is made between the normal and CA immune sera, and that absorption of CA antibodies substantially reduces the opsonic power of CA antiserum with *E. coli* O14 as test organism.

The moderate opsonizing capacity of normal rabbit serum probably is due to presence of CA antibodies in low titer, for such antibodies could be demonstrated by means of hemagglutination tests. In an attempt to replace rabbit serum with antibody-free, complement-containing serum, fetal calf serum, γ -globulin-free calf serum, and piglet serum were used. Normal fetal calf serum promoted phagocytosis moderately and was shown to contain CA antibodies in a titer of 1:10

in hemagglutination tests. γ -Globulin-free calf serum did not opsonize enteric bacteria, possibly because of lack of complement or of other factors. Piglet serum, which did not contain demonstrable CA antibodies, promoted phagocytosis, but to a lesser degree than did normal rabbit serum.

Attempts to determine whether intracellular killing of enteric bacteria takes place were unsuccessful, for almost all organisms had lost viability in the presence of leukocytes and normal rabbit serum, and significant differences between the latter and immune serum thus could not be demonstrated. It was deemed of interest, therefore, to extend the previous observations of Kunin and Beard (9) on the bactericidal effects of CA antibodies. These authors found that *E. coli* O14 serum was bactericidal to a slight degree only against the homologous organism but not against other enteric bacteria containing CA. Bactericidal tests were carried out with various enteric bacteria, CA antiserum produced by different methods, guinea pig complement, and heat-inactivated normal rabbit serum as diluent. The results of a typical experiment are presented in Table 3. It is evident that CA antibodies are bactericidal against *E. coli* O14, irrespective of the microorganism used for producing CA antibodies, but not against *S. typhimurium*. Additional tests were performed with *E. coli* O55, *E. coli* O111, and *S. choleraesuis*, and no evidence of killing was observed. It is clear, therefore, that CA antisera are not bactericidal for test organisms, containing CA, other than *E. coli* O14.

DISCUSSION

The present studies were undertaken to add to the limited knowledge of the biological significance of the CA of enteric bacteria and its antibody. It has been shown that CA antibodies opsonize enteric bacteria for phagocytosis by polymorphonuclear leukocytes of the rabbit. The specificity of the reaction was documented by the observation that, under identical conditions, *P. aeruginosa*, which does not contain CA, is not phagocytized to a greater extent by CA antiserum than by normal serum. In addition, absorption of CA antibodies reduces the opsonizing capacity of the CA antiserum. In view of the fact that phagocytosis is one of the major defense mechanisms of the host, it is conceivable that CA antibodies may play a protective role, particularly since CA antibodies have been demonstrated by means of hemagglutination tests in both rabbit and human serum (20). In addition, it has been shown previously that some patients with infection due to CA-containing bacteria respond with an increase in the titer of these antibodies (20). Had informa-

tion on the operative antigen-antibody system in the above-reported experiments not been available, enhanced phagocytosis might have been ascribed erroneously to nonspecific factors. Therefore, the results of the present investigation on the activity of antibodies against a widely distributed antigen deserve consideration in connection with the problem of cellular immunity (6, 8, 13) and of cytophilic antibodies (1, 14, 15). It will be interesting to learn whether cytophilic antibodies with CA specificity do occur. CA may represent only one of several antigens shared by enteric bacteria; for example, Brodhage (2, 3, 4) demonstrated another common antigen in urea extracts of various *Enterobacteriaceae*.

Study of the bactericidal action of CA antibodies has revealed that, in the presence of complement, they effect killing of *E. coli* O14 but not of other enteric bacteria sharing this antigen. Previously, Kunin and Beard (9) had shown that *E. coli* O14 antiserum is bactericidal only to the homologous serotype. The reason for this difference in susceptibility is not clear, and several possibilities may be considered. It is known that *E. coli* O14 produces considerably more CA than do other enteric bacteria, and the question arises whether differences exist also with regard to the amounts of CA on the surface of these bacterial cells. Furthermore, it has been shown that in *E. coli* O14 the molecule with CA specificity is ethyl alcohol-insoluble and resistant to a factor, presumably an enzyme, produced by *Pseudomonas* species; in contrast, in other enteric bacteria CA is ethyl alcohol-soluble and is susceptible to the *Pseudomonas* factor (21, 22, and Suzuki et al., Ann. Immunol. Hung., *in press*). Finally, the spacial relationship of CA and O antigen (lipopolysaccharide) may differ in *E. coli* O14 and in the other enteric bacteria, since previous studies suggest that lipopolysaccharides interact with isolated CA of the latter and, as a result, interfere with antibody formation (18).

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