Production of Common Antigen by Enteric Bacteria Grown in a Synthetic Culture Medium

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Enterobacteriaceae share a common antigen (CA). The present investigation was carried out to determine whether this antigen is produced by representative strains of *Escherichia*, *Salmonella*, and *Serratia* grown in a completely synthetic medium. For comparative purposes, antigen production by the same strains grown in infusion broth was determined. CA, as assayed by indirect hemagglutination and immunogenicity studies in the rabbit, was produced by the microorganisms cultured in both media. The amount of CA produced by the strains varied according to the size of microbial population and to the length of culture. With the exception of *E. coli* O14, the strains studied, on day 7, produced 1.4 to 8 times more CA in infusion broth than in the synthetic medium; with *E. coli* O14, the ratio of CA in the respective media was 96:1. *E. coli* O14, but not *E. coli* O111 or *S. typhimurium*, when grown in the synthetic medium, engendered CA antibodies upon intravenous injection into rabbits. Ethanol extraction of the latter two strains yielded an immunogenic ethanol-soluble antigen preparation. The ethanol-soluble fraction of *E. coli* O111 also induced a secondary response in animals primed with *E. coli* O111 or *S. typhimurium* cultures grown in the synthetic medium. It is concluded that CA produced in a completely synthetic culture medium has the same attributes as CA produced in infusion broth.

Kunin et al. (3) first discovered, by means of hemagglutination with *Escherichia coli* O14 anti-serum, a common antigen (CA) produced by more than 100 different *E. coli* O groups and by other *Enterobacteriaceae* grown on infusion agar. Also, these investigators demonstrated CA in each of 12 *E. coli* O groups, including O14, in a chemically defined liquid synthetic medium supplemented with vitamins and amino acids. Cultures of *E. coli* O14 grown on infusion agar readily engender antibodies against CA upon intravenous injection into rabbits, whereas cultures of other enteric bacteria fail to do so. The lack of immunogenicity of the latter microorganisms is due to the immunosuppressive influence of the simultaneously present endotoxic O antigen (12). After removal of O antigen, which is insoluble in ethanol, the ethanol-soluble CA is highly immunogenic (11) and only minimally toxic (2). It is likely that CA antibodies are of biological significance, because they opsonize heterologous enteric bacteria for phagocytosis by polymorphonuclear leukocytes (1). In addition, increased titers of CA antibodies have been observed in sera of children convalescing from enterobacterial

enteritis (14). Recently, it was reported that active immunization with CA protects rabbits against pyelonephritis experimentally induced with *Proteus mirabilis*, a CA-containing microorganism (1a). In view of these findings, the possibility arises that CA may be effective for immunization against various gram-negative bacteria. Highly purified CA is not yet available. Since isolation and chemical characterization of the antigen may be facilitated by growing the organisms in a completely synthetic culture medium, the present investigation was undertaken (i) to determine whether CA is produced under these conditions by representative strains of *Enterobacteriaceae* and, if so, (ii) to compare growth of the organisms and production of CA in the synthetic and artificial media, and (iii) to study the immunogenicity of the antigen produced in the synthetic medium.

MATERIALS AND METHODS

Microorganisms. The following smooth bacterial strains from our stock culture collection were used: *E. coli* serogroups O111, O14, and O55. *Salmonella*

enteritidis (CDC) was obtained from the late P. R. Edwards (Center for Disease Control, Atlanta, Ga.); *S. typhimurium* (C5S) was made available by G. B. Mackaness (Trudeau Institute, Saranac Lake, N.Y.); R. C. Bartlett (Hartford Hospital, Hartford, Conn.) supplied *Serratia marcescens* strains 15 and 20.

Culture media. All strains were maintained on Kauffmann's egg medium (Difco). Two culture media were employed: (i) Brain Heart Infusion broth (BHI) (pH 7.4; Difco), and (ii) the synthetic medium (SM) described by Perlmann et al. (6). The latter medium was prepared by dissolving the following in 1,000 ml of distilled water: 10 g of $\text{CH}_2\text{CHOH}\cdot\text{COONa}$, 1.0 g of NH_4Cl , 0.7 g of K_2HPO_4 , 0.12 g of Na_2SO_4 , and 0.02 g of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$; the pH was adjusted to 7.2 with 0.1 N NaOH. BHI and SM were distributed in tubes (10 ml) and flasks (100 ml) and sterilized by autoclaving for 15 min at 15 lb of pressure (121 C).

Inoculation. Tubes of SM were inoculated with the test microorganisms and incubated for 5 to 6 hr in a water bath at 37 C. Samples (1 ml) were transferred to flasks of BHI and SM; the flasks were incubated at 37 C. On days 2 and 7, 10 ml was removed and assayed.

Viable counts. The number of viable cells was determined by preparing 10-fold serial dilutions, from 10^{-1} to 10^{-8} , and transferring 1 ml of each dilution to Quebec-style disposable petri dishes (Will Scientific, Inc.). Molten Brain Veal Agar (Difco; 14 to 16 ml) was poured into each dish and mixed with the diluted culture. The plates were incubated at 37 C overnight, and the number of colonies was counted. Data are based on average counts of the two highest dilutions yielding isolated colonies.

Optical density. Turbidimetric determinations of total bacterial populations were performed by measuring the absorbancy of undiluted cultures at 690 nm, by using a Beckman DB-G spectrophotometer; undiluted media were employed as standards.

Preparation of antigens. Samples (10 ml) of cultures in BHI or SM, obtained on days 2 and 7, were heated at 100 C for 1 hr and centrifuged at 2 C ($23,500 \times g$) for 20 min; the decanted, clear supernatant fluid, hereafter identified as HKS, was frozen until assayed. The ethanol-soluble fractions of *E. coli* O111 and *S. typhimurium* (C5S) were prepared by a procedure described previously (11). Briefly, a mixture of *E. coli* O111 HKS or *S. typhimurium* (C5S) HKS (1 volume) and 95% ethanol (8.5 volume) was kept at 22 C for 18 hr. The mixture was centrifuged at 2 C ($23,500 \times g$) for 20 min, and the supernatant fluid was evaporated in a partial vacuum at 22 C. The resulting powder was dissolved to the original volume in distilled water. Hereafter, this ethanol-soluble fraction will be identified as ES.

Assay for CA. The amount of CA in HKS or ES was determined by the hemagglutination test described earlier (13). Briefly, rabbit red blood cells, as a 2.5% suspension, were washed three times. The sediment of these washed erythrocytes was resuspended to the original volume in antigen in twofold serial dilutions from 1:2 to 1:256. After incubation in a water bath at 37 C for 30 min, the cells were washed three times to remove unadsorbed antigen and resus-

pended to the original volume in the diluent. The antigen-treated erythrocytes (0.2 ml) were added to CA antiserum (0.2 ml) in twofold serial dilutions, from 1:40 to 1:2,560. To avoid interference by O antibodies, CA antiserum prepared against *E. coli* O111 ES was used with blood cells treated with *E. coli* O14 HKS, and CA antiserum against *E. coli* O14 suspension was employed with CA of the remaining strains. The mixtures of treated erythrocytes and CA antiserum were incubated in a water bath at 37 C for 30 min, centrifuged at $1,300 \times g$ for 2 min, and examined grossly for hemagglutination. The reciprocal of the highest dilution of antigen resulting in minimal hemagglutination with CA antiserum diluted 1:40 was recorded as the CA titer.

Immunogenicity determination. A group of two to four New Zealand white rabbits (female, 1.8 to 2.2 kg) was employed with each immunogen prepared from cultures in BHI or SM. Four 1-ml injections of the following preparations were given intravenously: *E. coli* O14 suspension, *E. coli* O111 HKS, and *S. typhimurium* HKS were injected in dilutions of 1:10 on days 0 and 2; dilutions of 1:5 on day 5; and dilutions of 1:3 on day 10. *E. coli* O111 ES and *S. typhimurium* ES were injected undiluted by the identical schedule. After a resting period of 9 days, each rabbit received a single intravenous injection (1 ml) of *E. coli* O111 ES (1:10) prepared from growth in SM. For control purposes, groups of rabbits received uninoculated BHI or SM by the same immunization schedule. Blood samples were obtained before, during, and after immunization; serum was separated and frozen until tested for CA antibodies.

Assay for CA antibody. Titers of CA antibodies were determined by the hemagglutination and hemagglutination-inhibition tests described previously (11). Briefly, serum (0.2 ml) in twofold serial dilutions from 1:20 to 1:5,120 was mixed with rabbit erythrocytes (0.2 ml) that had been modified with *E. coli* O86 HKS, diluted 1:10. After 30 min of incubation at 37 C and 2 min of centrifugation at $1,300 \times g$, the resulting hemagglutination was read grossly. The reciprocal of the highest dilution of serum resulting in minimal hemagglutination was recorded as the CA antibody titer. Specificity was confirmed by inhibition of hemagglutination by undiluted *E. coli* O14 HKS.

Diluent. Phosphate hemagglutination buffer (pH 7.3; Difco) was employed as the diluent throughout.

RESULTS

Seven strains of *Enterobacteriaceae* were grown in SM or in BHI. On days 2 and 7, supernatant fluids of heated suspensions were assayed for CA. In addition, viable bacterial counts and optical densities were determined on unheated samples of the suspensions. The results of two experiments, which were in good agreement with each other, are shown in Table 1. Six of seven strains grown in SM produced detectable amounts of CA by day 2; the amounts produced by all seven strains, by day 7, had increased further as did the corresponding optical densities. Growth was better with six of the seven strains in BHI than in SM;

S. enteritidis grew at least as well in SM as in BHI. The production of CA was more rapid and greater in BHI than in SM; with the exception of *E. coli* O14, the selected strains of *Enterobacteriaceae* produced averages of 1.4 to 8 times more CA by day 7 in BHI than in SM; *E. coli* O14 produced an average of 96 times more CA in the former than in the latter medium, probably due to the relatively poor growth of this strain in SM.

Results of studying the immunogenicity of CA, produced by various representative strains of *Enterobacteriaceae* in SM and BHI, are summarized in Table 2. It may be seen that *E. coli* O14 grown in SM was immunogenic upon intravenous injection into rabbits. In contrast, cultures of *E. coli* O111 and *S. typhimurium* failed to engender CA antibodies. It can be noted further that rabbits immunized with cultures of *E. coli*

TABLE 1. Relationship between concentration of common antigen and population of *Enterobacteriaceae* grown in a synthetic medium^a

Bacteria	Media ^b	Day 2						Day 7					
		CA ^c		No. of viable bacteria ^d		OD ^e		CA		No. of viable bacteria		OD	
<i>Escherichia coli</i> O111	BHI	128	128	93	100	0.95	1.05	128	128	51	69	1.17	1.43
	SM	10	10	4	6	0.04	0.06	64	128	18	26	0.31	0.41
<i>E. coli</i> O14	BHI	128	128	21	27	0.78	0.82	128	256	8	12	0.60	0.60
	SM	<2	2	1	3	0.02	0.06	2	2	1	3	0.03	0.09
<i>E. coli</i> O55	BHI	128	128	42	62	0.61	0.69	128	128	20	30	0.70	0.78
	SM	8	16	32	46	0.04	0.04	16	32	11	15	0.22	0.28
<i>Salmonella enteritidis</i> (CDC)	BHI	128	128	8	14	0.16	0.18	128	256	25	37	0.60	0.78
	SM	16	16	34	42	0.63	0.71	32	32	22	32	0.81	0.95
<i>S. typhimurium</i> (CDC)	BHI	128	128	80	96	1.91	1.97	128	256	41	55	1.91	1.99
	SM	16	32	36	46	0.08	0.14	64	128	30	46	0.76	1.00
<i>Serratia marcescens</i> (15)	BHI	64	256	108	130	1.30	1.38	128	128	39	55	0.85	1.09
	SM	4	16	17	25	0.06	0.06	16	16	39	55	0.18	0.28
<i>S. marcescens</i> (20)	BHI	64	64	96	126	1.27	1.91	64	64	39	49	0.97	1.07
	SM	8	16	22	34	0.05	0.09	16	16	28	38	0.20	0.32

^a Observations are the results of two experiments each.

^b Abbreviations used are: BHI, Brain Heart Infusion broth; SM, synthetic medium.

^c Titers of common antigen.

^d Values expressed $\times 10^7$.

^e Optical densities.

TABLE 2. Common antigen (CA) hemagglutinin responses of rabbits to *Escherichia coli* O14, *E. coli* O111, or *Salmonella typhimurium* grown in synthetic medium or infusion broth

Growth media ^b	Bacteria	Prepn	Mean CA hemagglutinin titers ^a				
			Day 0	Day 10	Day 19	Day 22	Day 24
BHI	<i>E. coli</i> O111	Supernatant fluid	<20	<20	<20	240	800
	<i>E. coli</i> O111	Ethanol-soluble fraction	<20	533	1,033	1,280	1,707
	None	Medium control	<20	<20	<20	<20	30
SM	<i>E. coli</i> O14	Suspension ^c	<20	1,600	— ^d	— ^d	— ^d
	<i>E. coli</i> O111	Supernatant fluid	<20	<20	<20	120	480
	<i>E. coli</i> O111	Ethanol-soluble fraction	<20	160	853	1,280	1,920
	<i>S. typhimurium</i>	Supernatant fluid	<20	<20	<20	100	240
	<i>S. typhimurium</i>	Ethanol-soluble fraction	<20	93	373	640	853
	None	Medium control	<20	<20	<20	<20	40

^a Primary immunization on days 0, 2, 5, and 10; secondary immunization on day 19.

^b Abbreviations used are: BHI, Brain Heart Infusion broth; SM, synthetic medium.

^c Heated for 1 hr at 100 C.

^d Not done.

O111 and *S. typhimurium* were specifically primed; there was a rapid and substantial secondary response to a subeffective dose of immunogenic CA in primed but not in unprimed rabbits that had received the uninoculated culture medium for control purposes. Furthermore, Table 2 shows that ethanol-soluble CA of *E. coli* O111 and *S. typhimurium* grown in SM readily engendered antibodies in unprimed rabbits. These findings parallel previous observations on CA produced by bacteria grown on infusion agar (3, 5, 11, 15).

DISCUSSION

Numerous *Enterobacteriaceae* share a CA, first described by Kunin et al. (3). These investigators were successful in demonstrating CA in cultures of *E. coli* O groups, including O14, prepared in a chemically defined minimal liquid medium supplemented with vitamins and amino acids. The aim of the present study was to assess CA production and immunogenicity in a completely synthetic culture medium. It was observed that all strains, representative of various genera, did form this antigen, although in lesser amounts than in infusion broth. This information is of importance for the following reasons. (i) Production of CA in the synthetic medium excludes the possibility of medium-derived antigenic determinants. (ii) Such preparations may be advantageous in attempts to purify and chemically characterize the antigen. (iii) If CA were to be employed for immunization to protect against enterobacterial infection, the antigen prepared from bacteria grown in the synthetic medium has obvious advantages.

The influence of constituents of artificial culture media on antigen content of bacteria has been established. Recently, Moody et al. (4) showed that peptone with blood-group activity transferred this activity to blood-group inactive bacteria. Furthermore, an immunological relationship has been shown between certain animal tissues and bacteria (6-10). The report that rat colon and *E. coli* O14 have the same or similar heat-stable antigenic determinants (6) suggests the possibility that CA is present in artificial media derived from animal tissues. Therefore, the observation that the infusion broth did not engender CA antibodies does not preclude the possibility that this antigen is present in this medium in subeffective amounts. In the current study, CA was identified in the ethanol-soluble fraction of *E. coli* O111 and *S. typhimurium* grown in a fluid medium composed of sodium lactate and inorganic salts only. In view of the fact that the synthetic medium per se is not immunogenic, the evidence provided is convincing that CA arises intrinsically

from the bacteria and not from components of the culture medium. The fact that *E. coli* O14, but not other *Enterobacteriaceae*, grown in the synthetic medium stimulated a CA antibody response in the rabbit is in accord with findings concerning cultures grown on infusion agar (3). Moreover, the current data, regarding the priming and boosting influence of CA produced in the synthetic medium, parallel previous findings (5, 15) on the immunogenicity of CA derived from bacteria grown on artificial medium. In these latter studies, it was shown that cultures or supernatant fluids of enteric bacteria other than *E. coli* O14, containing both CA and immunosuppressive O (lipopolysaccharide) antigen, prime rabbits for the production of CA antibodies in high titer after a single injection of a subeffective dose of isolated and immunogenic CA.

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