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Biochemical Basis of the Immunogenicity of the Common Enterobacterial Antigen

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Of the numerous members of the family *Enterobacteriaceae* only a few strains, notably *Escherichia coli* O14 and R mutants of the *E. coli* R1-core type, engender antibodies against the common enterobacterial antigen (CA) following immunization of rabbits with heated suspensions or culture supernatants; other members produce nonimmunogenic CA of identical serological specificity. The biochemical basis of the immunogenic properties of CA of the former strains was investigated by determining the relationship between the CA determinant and the lipopolysaccharide molecule. Lipopolysaccharides extracted from R mutants of the *E. coli* R1-core type or of *E. coli* O14 by the phenol-chloroform-petrol ether method contain the CA determinant, in contrast to extracts of other CA-producing R mutants. This is evident from the observation that only the former absorb CA antibodies, utilizing erythrocytes coated with alkali-treated lipopolysaccharide preparations. Based on the findings that CA of R mutants of *E. coli* R1-core type follows lipopolysaccharide during all purification steps and that alkali treatment increases its affinity for erythrocytes parallel to that of the lipopolysaccharide, it is concluded that the CA determinant either is part of the lipopolysaccharide molecule or is strongly complexed with it. It is suggested that this association between CA and the lipopolysaccharide of *E. coli* R1-core type and *E. coli* O14 accounts for the heat stability of the immunogenicity of CA of these unusual strains.

Immunochemical investigations carried out during the past few years on R mutants of *Salmonella*, *Escherichia coli*, *Shigella*, and other members of the family *Enterobacteriaceae* have clearly shown that there exists only a comparatively small number of different R cores (6, 10, 11). For example, all strains of *Salmonella* hitherto investigated produce an identical R core, which is present also in certain serotypes of *Arizona* (11). Thus far, three different R cores have been identified in R mutants of *E. coli*, designated as *E. coli* R1, R2, R3 (10, 11). The *E. coli* R3 core is present also in *Shigella flexneri* type 4b and the *E. coli* R1 core also in *Shigella sonnei* and *Shigella boydii* type 3 (G. Schmidt, Zentralbl. Bakteriol. Infektionskr. Hyg. Abt. Orig., *in press;* and H. Mayer and G. Schmidt, *in preparation*). Therefore, an identical R core may be shared by strains belonging to different serotypes, species, or even genera. As far as the distinctive R antigens are concerned, mutants with the *E. coli* R1 core do not cross-react with R2- and R3-core types nor with *Salmonella* Ra (11). The different R-core types of *Enterobacteriaceae* are characterized also by their distinct patterns of sensitivity to a set of selected R-specific phages (G. Schmidt, Zentralbl. Bakteriol. Infektionskr. Hyg. Abt. Orig., *in press*).

As originally reported by Kunin et al. (4, 5), most strains of *Enterobacteriaceae* produce a common antigen (CA), but only a few of these strains, notably *E. coli* O14 and, to a lesser degree, *E. coli* O56, O124, and O144, elicit antibodies following immunization in rabbits. Recent studies (7, 17) have revealed that *E. coli* R1, in contrast to the other R-core types, engender CA antibodies in high titers. The present study was carried out to elucidate the biochemical basis of the immunogenicity of highly immunogenic strains.

MATERIALS AND METHODS

Bacterial strains. R mutants used in this investigation were previously shown to possess complete R-core lipopolysaccharides (LPS) of different structures (7, 10, 11). The strains are listed in Table 1.

Cultivation of bacteria. The bacteria were grown in a laboratory fermentor at 37 C at a constant *p*H

TABLE 1. *Bacterial strains and corresponding R-core types*

R mutants (strain no.)	Derived from wild-type strains	Lipopolysaccharides (R-core type)	References
F470	*Escherichia coli* O8:K27 (E56b)	*E. coli* R1	10
3140	*Shigella boydii* type 3 (3141)	*E. coli* R1	G. Schmidt[a]
1664	*S. sonnei*	*E. coli* R1	7 and G. Schmidt[a]
F628	*Arizona* O9a, 9c (1495)	*Salmonella* Ra	11

[a] Zentralbl. Bakteriol. Infektionskr. Hyg. Abt. Orig., *in press*.

of 7.2 (9). The conditions for growth and the media used were described previously (10, 11).

LPS. LPS used for passive hemagglutination and antibody absorption were extracted by two different methods (2, 14). Preparations designated as LPS-S_3 were obtained by the hot phenol-water extraction procedure of Westphal et al. (14) and purified by three runs in the ultracentrifuge at 105,000 × *g* each for 4 hr. LPS designated LPS-GS (7) were obtained from certain R mutants by the phenol-chloroform-petrol ether (2:5:8, v/v/v) procedure, according to Galanos et al. (2). The LPS were centrifuged once at 105,000 × *g* for 4 hr.

Hemagglutination. Hemagglutination tests were carried out as described previously (11). For coating of erythrocytes with LPS, alkali-treated LPS-GS preparations were incubated for 1 hr at 37 C. For coating with CA, LPS-S_3 preparations containing the CA determinant were used without prior alkali treatment, and the incubation period was only 30 min. Human erythrocytes of blood group A were used throughout.

Antibody absorption. CA antisera were prepared in rabbits by immunization with the ethanol-soluble fraction of *Salmonella typhimurium*, as described previously (12), or with heated suspensions of the *E. coli* R1-type mutant 3140 obtained from *Shigella boydii* type 3 (17). Absorption was carried out as follows. Human erythrocytes (0.5 ml of washed sediment) were incubated with 2 mg of LPS (either alkali-treated preparations of LPS-GS or untreated LPS-S_3) in 1 ml of saline at 37 C for 1 hr. The cells were washed three times with 5 ml of saline and then suspended in 1 ml of saline solution. Antiserum in a dilution of 1:10 and in a volume of 1 ml was mixed with an equal volume of erythrocytes, and the mixtures were incubated at 37 C for 1 hr with repeated shaking. When indicated, absorption was repeated once or twice.

The term "heat stability of the immunogenic properties of CA" is used to indicate that heating of the strains at 100 C for 1 hr does not cause a significant decrease in immunogenicity of CA. Conversely, strains with "non-heat-stable immunogenic CA" lose CA immunogenicity, although not CA antigenicity, under these conditions (16, 17).

RESULTS

Differences in the immunogenicity among various *E. coli* R mutants were described in the preceding publication (17). The suggestion was made previously that the *E. coli* R1 core LPS is responsible for heat-stability of the immunogenic properties of CA (7), because it may serve as a suitable receptor for the CA moiety. The experiments to be described were undertaken to test this hypothesis with the following considerations in mind. LPS-S_3 preparations contain the CA determinant, and, upon incubation with erythrocytes at 37 C for 30 min, the cells, as revealed by hemagglutination, are specifically agglutinated by CA antibodies. Alkali treatment markedly enhances the erythrocyte affinity of LPS (O antigens) (1, 8), a procedure which usually destroys the erythrocyte affinity of CA (7). In contrast to LPS-S_3 preparations, extracts from R strains (not belonging to the *E. coli* R1-core type) obtained by the phenol-chloroform-petrol ether procedure according to Galanos et al. (2) usually do not contain the CA determinant, indicating that LPS and CA generally are independent and separable antigens. This conclusion is in accord with the previously reported separation of the two antigens by means of ethanol (12). The question whether CA is produced as an LPS-CA complex in highly immunogenic strains, i.e., *E. coli* R1 mutants F470 and 1664, was investigated by studying comparatively the LPS of R strains whose immunogenic properties of CA are either heat-stable (F470, 1664) or heat-labile (F628, *Salmonella* Ra). The results of the hemagglutination tests are shown in Table 2. Incubation of erythrocytes with untreated LPS-GS of the two strains did not result in modification of the cells by CA-antiserum nor by the respective R antisera, thus indicating that neither CA nor R-LPS was fixed to erythrocytes. In contrast, LPS-S_3 preparations containing unbound CA were markedly effective in coating erythrocytes with the CA determinant. If CA and LPS form a complex in the highly immunogenic strains of the *E. coli* R1-core type, then, it would be expected that this complex fixes to erythrocytes after suitable pretreatment. Both alkali and heat treatment are known (8) to enable LPS to fix effectively to indicator red blood cells. It may be seen from the data shown

TABLE 2. *Erythrocyte modification with common antigen (CA) by different lipopolysaccharide (LPS) preparations and the effects of their pretreatment by either alkali or heat*

Strains	Heat stability of the immunogenic properties of CA of the strains	LPS prepn	Pretreatment of LPS prepn	CA antibody[a] titers (reciprocal)
Escherichia coli O8⁻:K27⁻ (F470)	Stable	LPS-GS	None	<10
E. coli O8⁻:K27⁻ (F470)	Stable	LPS-GS	100 C, 1 hr	160
E. coli O8⁻:K27⁻ (F470)	Stable	LPS-GS	Alkali-treated	160
E. coli O8⁻:K27⁻ (F470)	Stable	LPS-S_3	None	1,280
Arizona 9a⁻, 9c⁻ (F628)	Labile	LPS-GS	None	<10
Arizona 9a⁻, 9c⁻ (F628)	Labile	LPS-GS	100 C, 1 hr	<10
Arizona 9a⁻, 9c⁻ (F628)	Labile	LPS-GS	Alkali-treated	<10
Arizona 9a⁻, 9c⁻ (F628)	Labile	LPS-S_3	None	1,280

[a] CA antiserum prepared with the ethanol-soluble fraction of *Salmonella typhimurium*.

TABLE 3. *Differences in antibody-neutralizing capacity of erythrocyte-attached, alkali-treated lipopolysaccharides (LPS) of different R-core types*

LPS-GS used for absorption			No. of absorptions	CA antibody titers (reciprocal)[a]	
Strains	R-core type of strains	Heat-stability of the immunogenic properties of CA of strains		*Shigella boydii* 3⁻ (R1 core)[b]	Ethanol-soluble CA of *Salmonella typhimurium*[b]
Escherichia coli O8⁻:K27⁻ (F470)	*E. coli* R1	Stable	2	80	40
E. coli O8⁻:K27⁻ (F470)	*E. coli* R1	Stable	3	60	
Shigella sonnei II (1664)	*E. coli* R1	Stable	2	60	40
Arizona 9a⁻, 9c⁻ (F628)	*Salmonella* Ra	Labile	1	2,560	1,280
Unabsorbed antisera			0	2,560	1,280

[a] *Salmonella greenside* R (1208); LPS-S_3 (LPS + CA) as indicator. CA, common antigen.
[b] CA antisera.

in Table 2 that, in fact, pretreatment (heat as well as alkali) of LPS-GS of strain F470 results in a significant hemagglutination by CA antibodies, whereas pretreatment of LPS-GS obtained from strain F628 failed to enhance hemagglutination. For control purposes, the capacity of the unheated, heated, and alkali-treated LPS-GS preparations to modify erythrocytes was determined utilizing homologous R1 and *Salmonella* Ra antisera, respectively. It was observed that the antisera failed to agglutinate even in a dilution of 1:10 red blood cells exposed to the untreated preparations, in contrast to the treated antigens, the antibody titers with the latter ranging from 1:1,280 to 1:2,560. It has been shown recently that the ability to coat erythrocytes with unbound CA, present in non-immunogenic strains, is lost during mild treatment with alkali, although its antibody-neutralizing capacity remains unaltered (7, 16). On the basis of the above findings, it is suggested that CA present in immunogenic strains becomes attached to erythrocytes because of its linkage to the LPS molecules.

It became of interest, then, to determine the CA-neutralizing capacity of alkali-treated LPS preparations obtained from different strains. To this end, antigenically modified erythrocytes were used for antibody absorption. The results of representative experiments are shown in Table 3. It may be seen that substantial neutralization of CA antibodies was accomplished by alkali-treated LPS-GS preparations of the F470 and 1664 strains producing heat-stable immunogenic CA. In contrast, antibody neutralization was not effected by alkali-treated LPS of strain F628 which produces non-heat-stable immunogenic CA. It is recognized that absorption with the former antigens does not remove all CA antibodies, although it does remove more than 90%. It is conceivable that the residual titer of CA antibodies is due to antibodies directed against the unaltered structure. Indeed, as shown in Table 4, free CA, fixed to

TABLE 4. *Effect of absorption of common antigen (CA) antisera with erythrocytes coated with phenol-water-extracted lipopolysaccharides (LPS-S$_3$)*

LPS-S$_3$ used for absorption	Heat stability of immunogenicity of CA in original strain	CA antibody titers (reciprocal)[a]	
		Shigella boydii 3$^-$ (R1 core)	Ethanol-soluble CA of *Salmonella typhimurium*
Absorbed with *Shigella sonnei* II (1664)	Stable	<20	<20
Arizona 9a$^-$, 9c$^-$ (F628)	Labile	<20	<20
Unabsorbed		2,560	1,280

[a] *Salmonella greenside* R (1208); LPS-S$_3$ (LPS + CA) as indicator.

erythrocytes, in the absence of LPS completely removes all CA antibodies by a single absorption. Differences among various LPS-S$_3$ preparations were not noted.

DISCUSSION

Since the original observation of Kunin et al. (4, 5), it has been known that CA of *Enterobacteriaceae* exists in two forms, one being highly immunogenic in the rabbit and the other, at best, poorly immunogenic. More recent observations have revealed that R mutants of *Enterobacteriaceae* also differ in the immunogenicity of CA which they produce. As reported previously (7, 17) *E. coli* R1-type mutants, when injected into rabbits in the form of heated (100 C for 1 hr) suspensions or of supernatants therefrom, engender CA antibodies in high titers. In contrast, *E. coli* R2- and R3-type mutants are ineffective under these conditions. *E. coli* R1 mutants, therefore, resemble *E. coli* O14, and the other R-core types resemble most other strains of *Enterobacteriaceae*. Another difference between CA of *E. coli* O14 and that of other S forms of *Enterobacteriaceae* was previously demonstrated (12, 13), the former being ethanol-insoluble and the latter soluble. *E. coli* O14, in addition to producing highly immunogenic ethanol-insoluble CA, also produces non-immunogenic ethanol-soluble CA. The present investigation has revealed that CA produced by the R1-type mutants of *E. coli* and *Shigella* also exists in two states. One kind of molecule is free, i.e., not bound to the LPS core; it is extractable with phenol-water, and it becomes attached without prior treatment to erythrocytes. This type of CA molecule appears to be common to most *Enterobacteriaceae*. The other moiety of CA produced by *E. coli* R1-type mutants appears to be complexed or to be part of the LPS, since it follows the latter in all purification steps: it is extracted with the LPS, using the phenol-chloroform-petrol ether extraction of Galanos et al. (2); it sediments together with LPS in the ultracentrifuge at 105,000 × *g;* and, like LPS, it becomes attached to erythrocytes only, or preferentially, after pretreatment with either alkali or heat. All these characteristics are in accord with the assumption that bound CA of *E. coli* R1-type strains is part of the LPS molecule. A mutant strain of *E. coli* O8: K27 (F614) with the R1 core of *E. coli* does not produce the LPS-linked CA, but only free CA as do other *Enterobacteriaceae* (7). This observation clearly indicates that the serological specificities of CA and *E. coli* R1-core antigen are different, and that the events linking CA to the *E. coli* R1-core (e.g., in strain F470), resulting in heat-stable immunogenic form of CA, are under genetic control. The major characteristics of the two CA moieties are summarized in Table 5.

Recently, Hammarström et al. (3) studied the immunochemistry of CA of *E. coli* O14 and concluded that it is part of the core structure of this microorganism. Thus, studies of *E. coli* O14 and of the *E. coli* R1-mutant type lead to similar conclusions. Indeed, it was shown previously (7) that the isolated LPS of *E. coli* O14 and *E. coli* R1 strains cross-react, indicating the presence of similar structures. In addition, recent experiments indicate that *E. coli* O14 represents an R form encapsulated by the K7 antigen (G. Schmidt and H. Mayer, *manuscript to be published*). This suggestion is based partly on the fact that one can extract LPS of *E. coli* O14 with phenol-chloroform-petrol ether according to Galanos et al. (2), an extraction method which is known to extract only LPS of R mutants but not the more hydrophilic LPS of S forms. Using the same absorption technique as described for the *E. coli* R1 mutants, erythrocytes coated with alkali-treated LPS-GS of *E. coli* O14 were found to absorb CA antiserum to the same extent as *E. coli* R1 LPS-GS preparations. This finding supports the assumption that the presence of certain R-core structures, such as that of *E. coli* R1, plays an important role in the immunogenic properties of CA (7, 17). The assumed R character of *E. coli* O14 may explain its rather unique position among the wild-type *Enterobacteriaceae*, as being one of the very few serotypes with heat-stable immunogenic CA.

In the preceding paper (17) it was shown that an encapsulated *E. coli* R1 mutant (F782)

TABLE 5. *Differentiation and characteristics of the common antigen (CA) in Escherichia coli R1 type mutant and in R mutants with complete R antigens of other R-core types*

Core type	Immunogenicity[a] of CA by immunization of rabbits		State of CA in the R mutants	Extractability		Ethanol solubility (85% aqueous solution)
	Heat-killed R mutants	Living R mutants		Phenol-chloroform-petrol ether	Phenol-water at 67 C	
E. coli R1	+++	++++	Bound to LPS (and free)	Extracted	Extracted	Insoluble (and soluble)
E. coli R2 and R3, *Salmonella* Ra	−	++	Free	Not extracted	Extracted	Soluble

[a] Symbols: − to ++++, various degrees of immunogenicity.

produces heat-stable immunogenic CA as does the K⁻ strain (F470). Similarly, the K7 antigen of *E. coli* O14 does not interfere with the immunogenic properties of the heat-stable CA of this strain. Thus, K antigens usually do not interfere with the immunogenicity of CA. The biochemical characteristics of the R core present in *E. coli* O14 are being investigated.

Finally, the studies reported here on the CA immunogenicity of various *Enterobacteriaceae* (17) and of R mutants may explain the presence of CA antibodies in normal subjects and increases in the titers of these antibodies in patients recovering from enterobacterial infection (15). The possible immunogenic role of R mutants emerging during infection deserves further investigation.

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