A NEW LIPOPOLYSACCHARIDE ANTIGEN IDENTIFIED IN *ACINETOBACTER CALCOACETICUS*: OCCURRENCE OF WIDESPREAD NATURAL ANTIBODY

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SUMMARY. Serological investigation of the lipopolysaccharide (LPS) of *Acinetobacter calcoaceticus* revealed a new antigen to which antibody in high titre is present in the serum of many mammalian species. The passive haemolysis test showed that antibody, in titres ranging from 32–4096, was invariably present in the serum of mice, rats, guinea-pigs, and horses. Rabbits and human beings had lower and more variable titres (<2–512). The antigen persisted after prolonged hydrolysis of the LPS in 1% acetic acid at 100°C. *Acinetobacter* lipid A, which resembled antigenically the lipid A of many gram-negative bacteria, could be distinguished from the new antigen by inhibition and absorption experiments. Antibody to the new antigen could be completely absorbed with *acinetobacter* lipid A but not with enterobacterial lipid A; moreover, the latter failed to react with the antibody in the passive haemolysis test. Immunisation of rabbits with lipid A-immunogenic *acinetobacter* cells gave rise to antibodies against the new antigen and to lipid-A antibodies. Absorption of the immune serum with *acinetobacter* lipid A removed antibody to both antigens, but absorption with enterobacterial lipid A removed only the lipid-A antibodies.

INTRODUCTION

Lipopolysaccharide (LPS) is a constituent of the outer membrane of gram-negative bacteria, conferring stability to the cell wall. LPSs influence pathogenicity by causing granulocytosis, fever, and haemodynamic changes leading to shock and death (Weinbaum, Kadis and Ajl, 1971). They are also potent antigens. All three regions of the LPS molecule—O polysaccharide, core, and lipid A—are immunogenic (Kauffmann, 1966; Lüderitz *et al.*, 1966; Galanos, Lüderitz and Westphal, 1971). The O polysaccharide and its antibodies have been investigated intensively (Kauffmann, 1966; Lüderitz *et al.*, 1966). O antibodies are specific for a given serotype. Core antigens, which are limited in number, are identical in many species. They have been defined chemically, but the biological relevance of their corresponding antibodies is uncertain (Lüderitz *et al.*, 1966; Chedid *et al.*, 1968; Ziegler *et al.*, 1973; Braude *et al.*,

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1977; McCabe *et al.*, 1977). Lipid A usually consists of a phosphorylated glucosamine disaccharide substituted with ester- and amide-linked fatty acids; little is known about lipid-A antibodies in terms of biological properties (Galanos *et al.*, 1977; Galanos, Lüderitz and Westphal, 1979), but they frequently cross-react with lipid A from bacteria belonging to heterologous gram-negative genera (Galanos *et al.*, 1971).

In a preceding paper, we described the isolation and purification of the LPS of *Acinetobacter calcoaceticus* (Brade and Galanos, 1982). The physico-chemical properties and the analytical data revealed an R-like LPS structure resembling that of rough mutants of Enterobacteriaceae. In this study, we report a new antigen in the LPS and lipid A of *A. calcoaceticus*, against which many mammalian species, including man, have naturally occurring antibodies.

MATERIALS AND METHODS

Lipopolysaccharide and lipid A. Strain NCTC10305 of *A. calcoaceticus* was used. The preparation of pure LPS in the uniform triethylamine or sodium salt form and of free lipid A was carried out as described earlier (Galanos *et al.*, 1979; Brade and Galanos, 1982). Enterobacterial lipid A from *Salmonella minnesota* strain R595 (Re) was also used.

Immune sera. Lipid-A antisera were obtained by immunising New Zealand White rabbits with acid-treated (1% acetic acid, 100°C, 1 h) *A. calcoaceticus* strain NCTC10305 according to the procedure of Galanos *et al.* (1971). Animals received 50, 100, 200, and 400 µg of lyophilised bacteria intravenously on days 0, 4, 7 and 11, respectively, and were bled on day 16. The serum collected was absorbed with sheep red-blood cells (SRBC) and stored at -20°C.

Normal sera. Sera from mice (strains NMRI, C3H/HeJ, C57BL/6, and NuNu), rats, guinea-pigs and rabbits were obtained from the animal stock of our Institute. Horse sera were kindly provided by Professor D. Hammer. Normal human sera were obtained from healthy blood donors, aged 20-65 years, of both sexes.

Serology. SRBC coated with alkali-treated LPS or lipid A, and guinea-pig complement, were used in the passive haemolysis and passive haemolysis inhibition tests, as described by Neter (1956) and Galanos *et al.* (1971). The release of free lipid A from LPS during hydrolysis was followed serologically by inhibition tests. Samples of 1 mg of LPS were hydrolysed in sealed ampoules in 200 µl of 1% acetic acid at 100 °C for different times, cooled in iced water, and diluted with 800 µl of distilled water; the pH was adjusted to 7.0 with triethylamine.

Absorption experiments. Undiluted antiserum (500 µl) was absorbed at 0 °C for 1 h with 100 µl of packed SRBC coated with either acinetobacter or salmonella alkali-treated lipid A, and centrifuged under refrigeration. Alternatively, antisera were fractionated on immunoabsorbent columns. Acinetobacter or salmonella lipid A which had been treated with hydrazine at 60°C for 2 h was coupled to AH-sepharose 4B (Deutsche Pharmacia, Freiburg) by means of glutardialdehyde (Galanos, unpublished data). Absorption of antibodies was carried out at room temperature in phosphate-buffered saline (PBS) by recycling the serum with a peristaltic pump. The column was washed thoroughly with PBS and the absorbed antibodies were eluted with 0.1 M glycine in HCl, pH 2.8, and collected in ice-cold PBS (10-fold ionic strength). The fraction which had not been absorbed by the column, and the eluted fraction, were concentrated to the original volume by vacuum dialysis and examined by the passive haemolysis test.

RESULTS

Serological properties

The antigenic similarity between acinetobacter and salmonella lipid A was investigated by the passive haemolysis and passive-haemolysis inhibition tests. The results are given in table I.

TABLE I

Reactivity of lipid A from *Salmonella minnesota* R595 (Re) and *Acinetobacter calcoaceticus* NCTC10305 with homologous and heterologous antiserum before and after absorption with homologous and heterologous lipid A-coated erythrocytes

Antiserum	Passive haemolysis-test titres when erythrocytes were coated with lipid A from	
	<i>S. minnesota</i>	<i>A. calcoaceticus</i>
Salmonella lipid A		
Before absorption	2048	2048
Absorbed with homologous lipid A	<2	<2
Absorbed with acinetobacter lipid A	<2	<2
Acinetobacter lipid A		
Before absorption	2048	25600
Absorbed with homologous lipid A	<2	<20
Absorbed with salmonella lipid A	<2	25600

Salmonella lipid-A antiserum reacted strongly with SRBC coated with either salmonella or acinetobacter lipid A. As in previous studies, salmonella lipid-A antiserum showed no interaction with SRBC coated with acinetobacter LPS (data not shown). In inhibition tests with salmonella lipid-A antiserum (table II), the lysis of SRBC coated with either acinetobacter or salmonella lipid A was completely inhibited by both types of lipid A. Moreover, absorption of salmonella lipid-A antiserum with salmonella or acinetobacter lipid A abolished completely its activity against both antigens (table I). From these results it is concluded that the lipid-A antigen present in all members of the Enterobacteriaceae is also present in *A. calcoaceticus*.

The use of acinetobacter lipid-A antiserum led to unexpected results. In the passive haemolysis test (table I), the acinetobacter lipid-A antiserum reacted with SRBC coated with either acinetobacter or salmonella lipid A, with titres of 25 600 and 2048 respectively. In the inhibition test (table II), acinetobacter lipid A inhibited the activity of the antiserum against both antigen preparations. Salmonella lipid A,

TABLE II

Inhibition of passive haemolysis by free lipid A from *S. minnesota* and *A. calcoaceticus*

Antigen-antibody* system	Minimal inhibitory concentration (μ g) of free lipid A from	
	<i>S. minnesota</i>	<i>A. calcoaceticus</i>
Salmonella lipid A and anti-salmonella lipid A	0.016	0.032
Acinetobacter lipid A and anti-salmonella lipid A	0.016	0.016
Acinetobacter lipid A and anti-acinetobacter lipid A	> 5.0	0.004
Salmonella lipid A and anti-acinetobacter lipid A	0.016	0.016

*Three haemolytic units of antiserum were used.

however, inhibited activity against SRBC coated with salmonella but not acinetobacter lipid A. Absorption of the antiserum with acinetobacter lipid A abolished completely its activity against both acinetobacter and salmonella lipid A. Absorption with salmonella lipid A, however, abolished activity against salmonella but not acinetobacter lipid A (table I). Similar results were obtained with lipid A from other Enterobacteriaceae (*Escherichia coli* and *Proteus* spp.; data not shown). These results show that acinetobacter lipid A contains, in addition to the already established antigen of lipid A, a new antigen; the latter is, however, absent from the lipid A of *S. minnesota* strain R595 (Re). Immunisation of rabbits with acid-treated acinetobacter cells leads to the production of antibodies against both antigens.

TABLE III

Fractionation of acinetobacter lipid-A antiserum with acinetobacter and salmonella lipid-A immunosorbent

Acinetobacter lipid-A antiserum	Passive haemolysis-test titres when erythrocytes were coated with lipid A from	
	<i>S. minnesota</i>	<i>A. calcoaceticus</i>
before absorption	2048	25600
after acinetobacter lipid-A immunoabsorption		
Unabsorbed fraction	< 2	< 20
Eluted fraction	1024	12800
after salmonella lipid-A immunoabsorption		
Unabsorbed fraction	< 2	25600
Eluted fraction	1024	2048

The presence of two different antigens in acinetobacter lipid A, and of their corresponding antibodies in acinetobacter lipid-A antiserum, was also demonstrated by immunoabsorption experiments. Antiserum to acinetobacter lipid A was fractionated on immunoabsorbent columns to which either acinetobacter or salmonella lipid A had been coupled. The fraction that did not bind to the immunoabsorbent, and the eluted antibodies were examined separately (table III). Both antibodies were absorbed by the acinetobacter lipid-A immunosorbent, and recovered almost completely by elution. The salmonella lipid-A immunosorbent bound those lipid-A antibodies that, after elution, reacted with SRBC coated with lipid A from both *Acinetobacter* and *Salmonella*. Antibody to the new antigen was not bound by the immunosorbent, and reacted only with SRBC coated with acinetobacter lipid A.

Rate of appearance of the two lipid-A antigens during acid hydrolysis of acinetobacter LPS

This was studied by measuring, by the passive haemolysis method, the inhibitory activity of hydrolysed LPS for the following antigen-antibody systems: salmonella lipid A with homologous antiserum, and acinetobacter lipid A with homologous antiserum. The figure shows that the intact LPS did not inhibit the salmonella system. This confirms the known masked character of the lipid-A antigen. After 3 min, a considerable amount of free lipid A was detected (inhibition value 250 ng). The

FIGURE—Rate of release of free lipid A from lipopolysaccharide of *Acinetobacter calcoaceticus* (NCTC10305) by hydrolysis in 1% acetic acid at 100°C. Free lipid A was estimated by its inhibition of the passive-haemolysis systems (1) salmonella lipid A and homologous antiserum (●), and (2) acinetobacter lipid A and homologous antiserum (O). Inhibition values are given in µg of hydrolysed lipopolysaccharide inhibiting 3 hemolytic units of antiserum.

maximal inhibition value (32 ng) was reached after 15 min. The release of 3-deoxy-D-manno-octulosonic acid (dOc1A) paralleled the release of free lipid A (data not shown). In contrast, in the acinetobacter system the intact LPS showed a significant inhibition value (125 ng). The maximal value (4 ng) was obtained after hydrolysis for 25 min. These results show that, unlike the established lipid-A antigen, the new antigen is already unmasked to some extent in intact LPS. Acid hydrolysis leads to its complete unmasking.

Occurrence of natural antibody to the new lipid-A antigen of A. calcoaceticus

The presence of antibody to acinetobacter lipid A in the serum of different animal species was studied by the passive haemolysis test, in which SRBC were coated with acinetobacter lipid A. As in all the experiments described above, fresh guinea-pig serum absorbed with acinetobacter lipid A was used as a source of complement, because, as will be shown below, normal guinea-pig serum is rich in antibody to the new lipid-A antigen.

Table IV shows the results. The highest antibody concentrations occurred in mice (including NuNu mice and the LPS non-responder strain C3H/HeJ; see Sultzer, 1972) and rats, in which titres of 1024–4096 and 512–1024, respectively, were found. Antibody was present in all individuals tested (60 mice and 30 rats), without significant

TABLE IV

Natural antibody to the new lipid-A antigen from A. calcoaceticus in different mammalian species

Species (n =)	Titres (passive haemolysis test)
Mouse, adult*(60)	1024-4096
newborn†(10)	32-64
Rat (30)	512-1024
Guinea-pig (40)	64-256
Horse (8)	512-2048
Rabbit (30)	< 2-256
Man (30)	< 2-512

* Strains C57BL/6, NMRI, C3H/HeJ, NuNu.

† Strain C57BL/6.

strain-dependent variation. In newborn mice (C57BL/6), the titres were lower (32-64) than in adults. Antibody was found in each of 40 guinea-pigs with titres of 64-256. Eight horse sera had titres of 512-2048. Antibodies also occurred in man and rabbits, but in these species the titres varied greatly (<2-512).

DISCUSSION

Serological examination of the lipid A of *A. calcoaceticus* revealed an antigen present also in the lipid A of members of the Enterobacteriaceae; and in addition a new antigen, to which natural antibodies are present in the serum of different mammalian species, including man.

The antigen of the well established type was demonstrated by the passive haemolysis test, in which antiserum against *S. minnesota* lipid A reacted equally with salmonella and acinetobacter lipid A absorbed to SRBC. All haemolytic activity was completely inhibited or absorbed by both acinetobacter and salmonella lipid A. The antigenic similarity between the lipid A of *A. calcoaceticus* and the members of the Enterobacteriaceae accords with chemical observations. In an earlier study, acinetobacter lipid A was shown to contain glucosamine 2 mol, amide-linked 2-hydroxylauric acid 2 mol, and esterbound fatty acids (lauric-, 2- and 3-hydroxylauric acid) 4 mol; phosphate and glucose were also present (Brade and Galanos, 1982).

For salmonella lipid A, the minimal structure that still retained full antigenic activity was shown to be β -1,6-linked glucosamine disaccharide carrying at least one amide-linked 3-hydroxymyristic acid (Galanos *et al.*, 1977). Although a glucosamine disaccharide has not been detected in acinetobacter lipid A, the presence of glucosamine and amide-linked 3-hydroxy fatty acids fulfils the compositional requirements for lipid-A antigenicity. As in the Enterobacteriaceae, this antigen is normally present in a masked form. In respect of the release of lipid A antigen and 3-deoxy-D-manno-octulosonic acid from acinetobacter LPS by hydrolysis, *A. calcoaceticus* resembles members of the Enterobacteriaceae.

The presence of a new antigen, of a hitherto unrecognised type, in the lipid A of *A. calcoaceticus* became apparent when antiserum to acinetobacter lipid A was examined in the passive haemolysis test with acinetobacter and salmonella lipid A adsorbed to SRBC. In the acinetobacter system the titre of the antiserum was higher (25 600) than in the salmonella system (2048). Activity against salmonella lipid A was inhibited or

absorbed by both antigen preparations, but that to acinetobacter lipid A only with the acinetobacter preparation. It should be noted that, because the antiserum's activity against salmonella lipid A was lower than that against the new antigen, at the antiserum dilution used in the inhibition test (3 haemolytic units) the salmonella lipid-A antibody was diluted out and played no rôle. The same was true for the absorption test, in which absorption with salmonella lipid A did not alter the overall activity of the serum against acinetobacter lipid A. It is interesting that, unlike the "classical" lipid-A antigen, which is expressed only in free lipid A after cleavage of the polysaccharide, the new antigen was already partly unmasked in intact acinetobacter LPS; it increased after removal of dOc1A by acid hydrolysis. In addition, the kinetics of the unmasking of each antigen during acid hydrolysis of acinetobacter LPS were distinctly different.

It is not yet known which structural component of acinetobacter lipid A accounts for the new antigen. As shown earlier, one main compositional difference between the salmonella and acinetobacter lipid A is the presence of glucose in the latter (Brade and Galanos, 1982). Although this sugar might appear an obvious candidate, it seems unlikely because we have recently found the new antigen under certain conditions in glucose-less LPS and LPS derivatives of gram-negative bacteria (including S-form bacteria) outside the family Neisseriaceae to which the genus *Acinetobacter* belongs (unpublished data).

An interesting biological finding of the present study is the ubiquitous presence of naturally occurring antibodies against the new antigen in the serum of man and all animal species investigated so far. Also interesting are the high concentrations of antibody in some species, particularly mice (including C3H/HeJ and NuNu mice), in which the titres ranged from 1024 to 4096; these may have been a reflection of the high number of plaque-forming cells found in the spleen (data not shown). Such titres are unusually high for natural antibodies (Galanos, 1972). Normal mouse serum does not contain classical lipid-A antibodies (Galanos *et al.*, 1977). Thus, normal mouse serum constitutes a readily available and rich source of antibody to the new antigen, and is at the same time free of antibodies to the "classical" lipid-A antigen.

Current unpublished studies suggest that the new antigen is also present in the LPS of many members of the Enterobacteriaceae and of other families of gram-negative bacteria.

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