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Protection Against Gram-Negative Infections with Antiserum to Lipid A from *Salmonella minnesota* R595

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The ability of antisera to lipid A, induced in rabbits by immunization with lipid A complexed to various carriers, to protect mice against gram-negative infection and to inhibit the fluid loss caused by an enteropathogenic strain of *Escherichia coli* in the piglet ligated gut was investigated. No significant protection was obtained in either case, although passive hemolysis and quantitative precipitation tests showed the presence of antilipid A antibodies in the sera. Fluorescent antibody studies suggest that the lipid A is in a cryptic position on the surface of smooth strains of gram-negative bacteria.

The importance of bacteremia and bacteremic shock is becoming widely recognized in hospital medicine. Infections caused by gram-negative bacilli constitute the largest category of hospital-associated infections. McCabe (16) has reported that bacteremia caused by gram-negative bacilli occurs with a frequency of 1 per 100 hospital admissions in which the mortality rate reaches 30%. Control of these infections has proved difficult, and survival depends more on the severity of the patient's underlying disease than the type of antibiotic treatment suggesting the need for additional control measures (5).

The increase in host resistance to various bacterial infections after administration of endotoxins is well known (for reviews see Shilo [25] and Rowley [24]). Cell walls of *Salmonella typhi* were shown to produce protection against *Escherichia coli* infection that reached a maximal level at 24 h and receded to normal after 5 days (22). Endotoxin (lipopolysaccharide [LPS]) was subsequently shown to be the active component (13, 23).

Studies of cell walls of gram-negative bacteria have demonstrated that most have almost identical LPS core structures (15). It therefore became of interest to determine whether an increase in resistance to infection could be produced by antiserum directed against these shared antigens.

Chedid et al. (2) had already reported that passive administration of antibody to a rough mutant of *S. typhimurium* protected mice against death after challenge with *Klebsiella pneumoniae*, and during the course of our work McCabe (17) established that active and passive administration with certain rough mutants of *S. minnesota* afforded heterologous protection to mice. The most deficient mutant (Re), in which the LPS was represented only by ketodeoxyoctonate (KDO) and lipid A, induced the greatest degree of protection.

Since lipid A is a common component of all LPS and is structurally identical or similar in all *Enterobacteriaceae* (14), we decided to study the ability of antibodies to lipid A to passively protect mice against heterologous challenge with gram-negative organisms and also to evaluate the protective capacity of lipid A antibodies to inhibit fluid loss caused by enteropathogenic *E. coli* in young pigs.

It has recently been shown (7) that it is possible to produce antibodies to lipid A by using acid-hydrolyzed *S. minnesota* R595 bacterial cells that contain free lipid A in their outer cell membranes. Higher titers of anti-lipid A antibodies were produced when these hydrolyzed bacteria were coated with additional lipid A. In the present study, antisera were raised to cell-free immunogens to avoid the presence of antibodies to cell wall components other than lipid A. Such antisera also facilitated the investigation of accessibility of lipid A on the bacterial surface.

MATERIALS AND METHODS

Bacteria. The *Salmonella* mutants used in this study were obtained from Otto Westphal, Max Planck Institute für Immunobiologie, Freiburg, West Germany. The bacteria were cultivated on the medium of Gmeiner et al. (10).

Challenge organisms. The organisms used for challenge in the mouse protection tests were obtained from P. Hunter of our chemotherapeutic department. These were *S. typhimurium* 33 U9 (human) 4M5182, *E. coli* O15.K?, and *Klebsiella* 18 "pneumoniae," untyped, capsulated, clinical isolate. The strain of *E. coli* used for the ligated gut experiments in baby pigs was an Abbotstown strain O149K88a,c, originally obtained from W. J. Sojka, Central Veterinary Laboratory, Weybridge, England.

Animals. White male mice (LACA from Oxford Laboratory Animal Centre, Oxford, England) weighing 18 to 20 g were used in all experiments. They were housed in groups of 10 per cage, with free access to food and water. After challenge they were inspected daily and the deaths were recorded.

Mouse protection tests: intraperitoneal route. Mice were injected with 0.25 ml of antiserum intraperitoneally (i.p.). Four hours later they were challenged i.p. with 0.5-ml volumes of organisms suspended in 0.15 M saline. The inocula were prepared by diluting log-phase cultures of organisms prepared by incubation in nutrient broth for 4 h at 37 C. The mean lethal dose of the strains was determined by the method of Reed and Muench (20) and was defined as the amount of culture killing 50% of normal control animals within 72 h.

Intravenous route. Infection was established and the mean lethal dose was determined by injection in the tail vein of 0.2-ml volumes of organisms suspended in 0.15 M saline. In the protection experiments, antiserum was given intravenously (i.v.) in a volume of 0.2 or 0.3 ml for 1 h before challenge as described in the text.

Lipid A preparation. LPS was extracted from acetone-dried *S. minnesota* R595 bacteria by the phenol-chloroform-petroleum ether method (6). Free lipid A was obtained by hydrolysis of the LPS according to the method of Risse et al. (21) and was solubilized by addition of triethylamine (8). Alkali-treated lipid A was prepared by heating free lipid A in 0.25 N NaOH at 56 C for 1 h. After removal of insoluble material, the supernatant was neutralized with acetic acid, dialyzed, and freeze-dried. Alkali-treated glycolipid was prepared in a similar manner.

Preparation of immunogens. To stimulate antibodies to lipid A, acid-treated *S. minnesota* R595 bacteria were coated with lipid A (7). A variety of protein carriers was also used for complexing with lipid A. The carriers used were bovine serum albumin (BSA; Armour Pharmaceutical Co. Ltd.), histone (from calf thymus; Sigma Chemical Co.), edestin (Sigma Chemical Co.), keyhole limpet hemocyanin (Calbiochem), and methylated BSA (Sigma Chemical Co.). The method used was an adaptation of the procedure used by Galanos et al. (9) for the preparation of BSA-lipid A. Briefly, lipid A (10 mg) in water (5 ml) was solubilized by addition of triethylamine (5 μliters). This solution was mixed with a solution of the protein carrier (10 mg) in water (5 ml). The resulting mixture was dried in a rotary evaporator under reduced pressure, whereby triethylamine was removed and complexing was allowed to take place. The complex was redispersed in distilled water and frozen.

Immunization schemes. (i) Rabbits: antisera prepared by the i.v. route. Rabbits received four i.v. injections of each immunogen on days 1 (100 μg), 4 (200 μg), 7 (300 μg), and 11 (500 μg). Animals were bled on day 16 by cardiac puncture.

Antisera prepared by the subcutaneous route. The antigen (1.5 mg in 1 ml of saline) was mixed with an equal volume of either Freund complete or incomplete adjuvant (Difco). A 0.5-ml amount was injected subcutaneously on each flank, and 4 $\times$ 0.2-ml injections were given intradermally. Rabbits were boosted 7 weeks later by repetition of the preceding schedule, and blood was taken 20 days later.

(ii) Mice. Mice were immunized i.p. with 0.1 ml containing 500 μg of BSA-lipid A in Freund complete adjuvant. Animals were boosted i.p. on day 10 with 100 μg of antigen in saline and bled 7 days later. Another group of mice was immunized with acid-hydrolyzed cells (AHC)-lipid A (2:1) i.p. Each mouse was given 0.1 ml containing 50 μg of antigen in Freund incomplete adjuvant. Animals were boosted at weekly intervals with 50, 150, and 250 μg of antigen i.p. in saline and bled 10 days later.

(iii) Goats. Two goats were immunized with 5 mg of AHC-lipid A (10:1) in 1 ml of saline and 1.5 ml of Freund complete adjuvant. A 1-ml amount was administered subcutaneously, and 5 $\times$ 0.2 ml was given intradermally. One goat was immunized with 5 mg of BSA-lipid A in 1 ml of saline and 1.5 ml of Freund complete adjuvant, and one goat was given Freund complete adjuvant alone as a control. The animals were boosted 6 weeks later with a repeated dose, and bleedings were taken weekly.

Passive hemolysis test. This method was used for determining anti-lipid A antibodies. Sheep erythrocytes (SRBC) were washed in phosphate-buffered saline (PBS) and sensitized in PBS at 37 C for 30 min with 60 μg of alkali-treated lipid A and 0.2 ml of packed SRBC. To each well containing 0.05 ml of diluted serum, 0.05 ml of a 0.5% suspension of sensitized SRBC and 0.05 ml of complement (1:40 dilution of guinea pig serum) were added. The plates were incubated at 37 C for 60 min and overnight at 40 C. The final dilution of serum producing 100% hemolysis was taken as the antibody titer of the serum. All heat-treated sera were preabsorbed with SRBC.

Passive hemagglutination test. Serial 0.05-ml twofold dilutions of antiserum were prepared in microtiter plates. To each well, 0.05 ml of a 0.5% suspension of sensitized erythrocytes was added, and the plates were incubated for 1 h at 37 C. Results were read after overnight incubation at 4 C. The end point was read as the highest dilution giving complete agglutination. This test was used to determine the antibody content of antiserum raised to the *E. coli* strain used for infecting mice. The antigen used for sensitizing the SRBC was obtained from bacteria grown overnight in nutrient broth (10 ml). After centrifugation the bacteria were resuspended in 10 ml of saline, boiled for 2.5 h, and centrifuged, and the supernatant was removed. One milliliter of 95% ethyl

alcohol was added to the supernatant, and a 1:10 dilution of this preparation was used to sensitize the SRBC.

2-Mercaptoethanol treatment of serum. The procedure of Chan and Deutsch (1) was followed. Samples were dialyzed against PBS containing 0.1 M 2-mercaptoethanol for 48 h. This was followed by dialysis against 0.02 M iodoacetamide for 48 h and finally dialysis against 0.9% NaCl for 48 h. Samples were then tested for antibody activity in the passive hemolysis test. Controls were treated in a similar manner, omitting the ME from the dialysis buffer.

Quantitative precipitation test. Tests were carried out by adding 0.2 ml of 0.9% NaCl containing various amounts of test antigen to 0.2 ml of undiluted antiserum. The tubes were incubated at room temperature for 30 min and overnight at 4 C. After centrifugation, the precipitates were dissolved in 2 ml of 0.1 N NaOH containing 2% Na_2CO_3, and the absorbance was read at 280 nm. The supernatant solutions were tested for residual anti-lipid A antibody by the passive hemolysis technique.

Fluorescent antibody technique. Smears of bacterial suspensions were air dried and fixed in ice-cold acetone for 10 min. Each preparation was flooded with test sera, diluted to the required concentration in PBS, and incubated in a humid chamber at room temperature for 30 min. Serum was drained off and slides were washed in 1 liter of buffered saline gently agitated by a magnetic stirrer. After draining, the preparations were flooded with fluorochrome (sheep anti-rabbit immunoglobulin labeled with fluorescein isothiocyanate; Wellcome Reagents Ltd., England) and again incubated for 30 min at room temperature in a humid chamber. After exhaustive washing in PBS, slides were drained and mounted in buffered glycerol.

Slides were viewed under oil immersion in a Zeiss WL microscope using a tungsten light source (optical system: 60-W tungsten bulb; KP 500/2 exciter; 2 × BG 38/2.6 and BG 12/2 for red suppression; no. 53 barrier filter; ×100/1.25 objective).

Ligtaed gut test in baby pigs. The method of Smith and Halls (26) was followed. Weaned 12-week-old pigs that had been starved before operation were anesthetized and the small intestine of each, beginning 1 m from the pylorus and ending 3 m from the pylorus, was divided into segments 10 cm long separated by 5-cm interloops by means of surgical ligatures. One milliliter of test serum or normal rabbit serum was incubated with an equal volume of 5 × 10^8/ml *E. coli* (Abbotstown strain) at 37 C for 1 h. A 0.1-ml amount was injected into alternate 10-cm segments of ligated pig intestine and the 5-cm interloops were left as untreated controls. The abdomen was closed with nylon sutures, and the pigs were allowed to recover from anesthesia. The animals were killed 20 h later by i.v. administration of pentobarbitone sodium, and the volume (in milliliters) and length (in centimeters) of treated segments were noted. Materials to be compared were always tested in segments close to each other because of variability in the reactivity of the intestine. Specific antiserum to the Abbotstown strain was prepared in rabbits by standard techniques (4) and used in the test as a positive control.

RESULTS

Anti-lipid A content of various immune sera. Rabbits were initially immunized with the bacterial immunogens used by Galanos et al. (7) according to the schemes described in Materials and Methods, and the anti-lipid A content of the sera was measured by the passive hemolysis technique.

Immunization with intact *S. minnesota* R595 bacteria resulted in low levels of anti-lipid A antibody. Higher levels were obtained by acid treatment of these cells to remove the KDO, and the highest titers were obtained when these hydrolyzed bacteria were coated with additional free lipid A. The soluble complex BSA-lipid A also gave high titers of anti-lipid A antibody when injected with Freund adjuvant.

Attempts to raise comparable levels in mice and goats were unsuccessful. Mice immunized with BSA-lipid A as described in Materials and Methods did not give rise to any anti-lipid A antibodies detectable by passive hemolysis. Mice immunized with AHC-lipid A gave reciprocal titres of 32. Goats immunized with BSA-lipid A or AHC-lipid A did not show any rise in titer of anti-lipid A antibodies over a control goat given adjuvant alone. All goats had detectable levels of anti-lipid A antibodies before immunization. The goat immunized with BSA-lipid A had levels of BSA antibodies in excess of 1:4,096 as determined by passive hemagglutination, whereas the preinoculate bleeding was negative for antibody to BSA.

Passive protection of mice with immune rabbit sera. Antisera with anti-lipid A titers of 512 or higher were tested for their ability to protect mice against challenge with a virulent strain of *E. coli*. Relevant control sera were also tested. No specific protection was afforded by the various immune sera compared with the protection afforded by the sera obtained from rabbits given adjuvant alone (Table 1). The good protection obtained with the latter sera was a consistent observation.

Attempts to raise anti-lipid A antibody with lipid A complexed to various protein carriers. The sera in Table 1 that contained anti-lipid A activity gave no protection, but this may have been because the titer was too low. Alternative carriers to BSA were complexed with lipid A and used to immunize rabbits in efforts to boost the titer of anti-lipid A anti-

body. A further modification was introduced in that the sera were unheated before the protection experiments in view of the observation by Galanos (personal communication) that in some human sera the antibody of lipid A specificity is heat labile.

Sera raised by the i.v. route, showing the highest titer of anti-lipid A antibodies, were tested for their protective effect in mice against *E. coli* O15 infection, and these results were compared with the effect obtained by using specific anti-*E. coli* O15 antiserum. Once again no significant protection was afforded compared with that obtained with normal rabbit serum. The protection obtained with specific anti *E. coli* O15 antiserum was evident (Table 2).

The inability of anti-lipid A serum to confer protection was also evident in mice challenged with *K. pneumoniae* 4 h after administration of serum. There was no increase in protection when compared with normal rabbit serum in mice passively immunized with antisera containing high levels of anti-lipid A antibodies (Table 3).

Passively immunized mice were also challenged with *S. typhimurium* as another example of a gram-negative infection, but once again the inability of the antiserum compared with normal rabbit serum to protect against challenge was evident (Table 4).

Passive protection of mice with immune serum given intravenously. Following the observation of McCabe (17) that antiserum to the Re mutant conferred protection when given i.v. against i.v. challenge, anti-lipid A antiserum was given i.v. to mice either 4 or 1 h before i.v. challenge with *K. pneumoniae*. There was little obvious protective effect of the anti-lipid A serum either given 1 or 4 h before challenge (Table 5).

Attempted inhibition of fluid loss caused by enteropathogenic E. coli by preincubation with anti-lipid A serum in the ligated gut test in piglets. Preliminary investigations showed

TABLE 1. *Protective effect of anti-lipid A antisera in mice on lethal E. coli 015 infection*

Serum from rabbits immunized with:	Anti-lipid A titer	Survivors[a] (%) after:			
		3 days		6 days	
		2 LD_{50}[b]	4 LD_{50}	2 LD_{50}	4 LD_{50}
AHC[c] in FICA[d]	512	90	30	80	20
AHC-lipid A in FCA[e]	1,024	100	50	80	30
AHC-lipid A in FICA	1,024	90	30	80	20
BSA-lipid A in FCA	512	80	40	70	30
FCA alone		100	70	80	60
FICA alone		80	40	70	30
Saline control		30	20	20	10

[a] Ten mice per group.
[b] Mean lethal dose.
[c] Acid-hydrolyzed cells of *S. minnesota* R595.
[d] Freund incomplete adjuvant.
[e] Freund complete adjuvant.

TABLE 2. *Protective effect of unheated anti-lipid A antisera in mice on lethal E. coli 015 infection*

Protective treatment	Anti-lipid A titer	72-h survivors[a] (%) at challenge doses of:		
		3 LD_{50}[b]	6 LD_{50}	9 LD_{50}
Saline		30	10	0
Unheated normal rabbit serum		70	40	10
Unheated methylated BSA-lipid A antiserum	4,096	70	60	20
Unheated KLH[c]-lipid A antiserum	2,048	80	40	0
Unheated Edestin-lipid A antiserum	1,024	30	20	20
Unheated histone-lipid A antiserum	4,096	80	60	20
Specific anti-*E. coli* antiserum	4	100	70	70

[a] Ten mice per group.
[b] Mean lethal dose.
[c] Keyhole limpet hemocyanin.

that it was possible to induce fluid accumulation into ligated gut segments of piglets on injection of 0.1 ml of varying dilution of an Abbotstown strain of *E. coli*, as first reported by Smith and Halls (26). However, a distinct variation in response between pigs and in reactivity of different segments was also noted. To minimize these differences, test sera and control serum (normal rabbit serum) were incubated with a standard amount of *E. coli*, and after incubation 0.1-ml samples were injected into ligated segments (Table 6). In pigs 1 and 2, antiserum specific for the *E. coli* inhibited the action of the microorganisms. Anti-lipid A serum had virtually no effect when tested in pigs 3 and 4, and in pig 5, where the two sera were directly compared with normal rabbit serum, the same result was obtained.

Fluorescent antibody studies. The availability of lipid A antigen on the bacterial cell surface was studied by indirect immunofluorescence. Methylated BSA-lipid A antiserum reacted with AHC-lipid A and with *S. minnesota* R595 but not with viable *E. coli* O15 or a suspension of *E. coli* O15 previously boiled for 1 h.

By using a series of *S. minnesota* mutants, it was possible to show a progressive increase in antibody binding of the methylated BSA-lipid A antiserum with increasing exposure of the lipid A site, the strongest reaction being obtained with R595 (Re), in which lipid A has only the KDO trisaccharide of the bacterial cell wall attached.

TABLE 3. *Protective effect of anti-lipid A antisera in mice on lethal Klebsiella pneumoniae infection*

Protection treatment	72-h survivors[a] (%) at challenge doses of:		
	1 LD_{50}[b]	5 LD_{50}	10 LD_{50}
Saline	20	10	0
Unheated normal rabbit serum	60	50	20
Methylated BSA-lipid A serum	50	40	20
Histone-lipid A serum	80	30	20

[a] Ten mice per group.
[b] Mean lethal dose.

Removal of anti-lipid A antibody by quantitative precipitation test. Antiserum containing antibodies to lipid A raised in rabbits to AHC-lipid A was mixed with increasing quantities of BSA-lipid A, alkali-treated lipid A, alkali-treated glycolipid, glycolipid, and BSA, and after precipitation, the supernatant fluids from the tubes showing maximal amounts of precipitate were tested for residual anti-lipid A antibodies by passive hemolysis (Fig. 1). At the point of maximal precipitation, only the tubes in which BSA-lipid A and alkali-treated lipid A had been added were the supernatants negative for anti-lipid A antibodies.

ME treatment of sera. Various antisera containing antibodies to lipid A were subjected to reduction with ME and subsequent alkylation with iodoacetamide, and the sera were tested for antibodies to lipid A by the passive hemolysis test. ME-treated sera were negative for lipid A antibodies after treatment.

DISCUSSION

The need for more effective methods for the control of infections caused by gram-negative bacilli has been stressed recently by McCabe et al. (19). Although there are a large number of species and serotypes that can produce these infections, all the organisms contain LPS, the lipid portion of which is known to be structurally identical or similar in all *Enterobacteriaceae* (14). The development of mutant strains of organisms that synthesize defective LPS containing only lipid A and KDO made it possible to isolate lipid A and study its biological activity (6).

Following the original observation of Galanos et al. (7) that it was possible to produce antibodies against lipid A, we decided to test the protection afforded by these antibodies against gram-negative infections.

TABLE 4. *Protective effect of anti-lipid A antiserum in mice infected with about 1 mean lethal dose (5 days) of Salmonella typhimurium intraperitoneally*

Protective treatment	Cumulative mortality (10 mice/group)													
	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14
Saline	0	0	0	1	3	4	9	9	9	9	9	9	9	9
Unheated normal rabbit serum	0	0	0	0	0	0	1	1	1	1	4	4	4	4
Unheated MeBSA/Lipid A serum	0	0	0	0	2	2	2	2	2	2	3	4	4	4

[a] Day after challenge.

The results reported in this paper tend to discount any beneficial role to antibodies to lipid A in protection against gram-negative infections. Although it has been possible to produce antibodies against lipid A by complexing it to various protein carriers, these antibodies do not afford protection against enterobacterial infections.

The various anti-lipid A antisera tested did not enhance the survival time of mice infected with either *E. coli*, *K. pneumoniae*, or *S. typhimurium* over that obtained with normal rabbit serum. The ability of the latter to protect some mice was a consistent observation throughout this study. Attempts to inhibit the enteropathogenic effect of a strain of *E. coli* in the pig ligated gut system by preincubation with anti-lipid A serum also proved negative.

Although no protective activity has been noted with antiserum to lipid A, Galanos et al. (7) reported that anti-lipid A antibodies sensitized *E. coli* for i.p. phagocytosis, and it is interesting in this respect that antibodies to another shared antigen of *Enterobacteriaceae*, Kunin's common antigen (12), have also been shown to opsonize heterologous gram-negative bacilli for phagocytosis (3), but the protection afforded mice challenged with *S. typhimurium* by antiserum to this common antigen has not been very striking (11). More recently, McCabe and Greely (18) failed to show any protective effect of passively administered rabbit antisera to common antigen against gram-negative infection in mice.

In earlier studies by Chedid et al. (2), showing protection against *K. pneumoniae* infection with passively administered antibody to a Re mutant of *S. typhimurium*, and recently by McCabe (17), with passively administered antibody to a Re mutant of *S. minnesota* showing protection against *E. coli* and *K. pneumoniae*, the antisera were raised against the whole mutant cells. The anti-lipid A titer of the sera

TABLE 5. *Protective effect of anti-lipid A sera given intravenously in mice challenged with Klebsiella pneumoniae intravenously*

Treatment	Survivors[a] (%)			
	Serum given 1 h before challenge		Serum given 5 h before challenge	
	3 days	6 days	3 days	6 days
Saline	45	0	55	10
Normal rabbit serum	45	5	70	30
Anti-lipid A serum	60	0	80	30

[a] Ten mice per group.

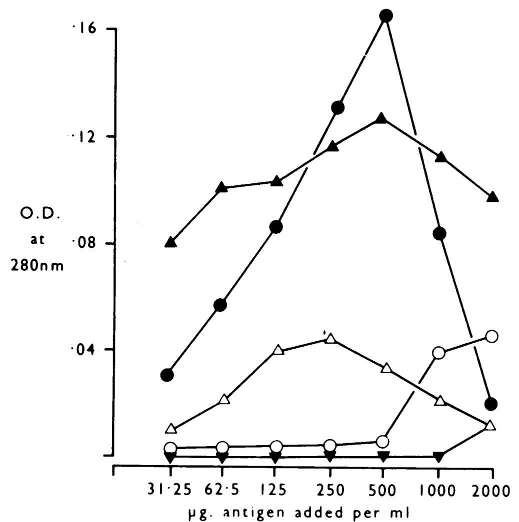

FIG. 1. *Quantitative precipitation tests on anti-lipid A serum with different antigens. Symbols: ●, BSA-lipid A; ▲, alkali-treated lipid A; △, alkali-treated glycolipid; ○, glycolipid; ▼, BSA.*

TABLE 6. *Effect of specific anti-Abbotstown serum and anti-lipid A serum on fluid accumulation in the ligated gut of piglets caused by an enteropathogenic Abbotstown strain of E. coli*

Pig no.	Test serum	Reduction (%) of volume/length ratio of test serum compared with normal serum in adjacent pairs of segments						
		1[a]	2	3	4	5	6	7
1	Specific anti-Abbotstown serum	100	80	100	100	71	100	100
2	Specific anti-Abbotstown serum	100	100	100	100	100	100	100
3	Anti-lipid A serum	3	33	0	11	0	0	0
4	Anti-lipid A	0	22	0	0	0	42	0
5	Specific anti-Abbotstown serum	0	48	100	71	71		
	Anti-lipid A serum	0	56	0	9	14		

[a] In pigs numbers 1 through 4, each column compares the effect obtained in paired segments numbered anterior to posterior, and in pig number 5 each column compares the effect obtained in triple segments.

was not reported. From our present work it would seem that the cross-reactive antigen responsible for the protection on the bacterial surface in both these cases is unlikely to be lipid A.

The fluorescent antibody studies suggest that lipid A is not sufficiently exposed on the bacterial surface, since lipid A was not detectable on the surface of heat-killed or viable cells of the smooth strain of *E. coli* used in the challenge experiments. That this is due to inaccessability rather than the relative amount of lipid A is supported by the results obtained by using the series of *S. minnesota* mutants, which show a progressive increase in antibody binding with increasing exposure of the lipid A site. The data from the quantitative precipitation test seem to confirm this suggestion. The amount of antibody precipitated by using glycolipid is much less than that obtained by using BSA-lipid A.

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