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Relationship of Structure to Function in Bacterial Endotoxins: Serologically Cross-reactive Components and their Effect on Protection of Mice against some Gram-negative Infections

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SUMMARY

Rabbit antisera were prepared against the heptoseless Re mutants, *Salmonella minnesota* R595 and *S. typhimurium* SL1102, as well as against purified R595 glycolipid coated on autologous erythrocytes. The antisera cross-reacted with the endotoxic glycolipids extracted from Re mutants of various bacterial strains, including *S. minnesota* R595, *S. typhimurium* SL1102, *Escherichia coli* D31m4, *E. coli* D21f2 and *E. coli* F515, as shown by passive haemagglutination and gel diffusion tests. The anti-Re sera also cross-reacted with the RESI preparations (a purified 'lipid A' fraction) from the endotoxic lipopolysaccharides of various heterologous smooth Gram-negative bacteria including *Serratia marcescens*, *Pseudomonas fluorescens* and *E. coli* 0127. However, the same antisera failed to protect mice against infection by Gram-negative bacteria such as *Klebsiella pneumoniae* type II, *S. typhi* 0901, *P. aeruginosa* 119 and *E. coli*. The results suggest that although the lipid moieties of the lipopolysaccharides in the cell wall of Gram-negative bacteria share cross-reactive immunodeterminant groups, these groups may not be accessible to antibody against them.

INTRODUCTION

Owing to the progressively increasing frequency of hospital-acquired Gram-negative infections (Finland, 1970), there have been several studies on the activities of antibodies to endotoxins and to cross-reactive bacterial antigens (Chedid *et al.*, 1968; McCabe, 1972; Ziegler *et al.*, 1973; Mullan *et al.*, 1974).

Nowotny, Radvany & Neale (1965) first reported that certain activities may be neutralized *in vitro* with homologous hyperimmune antisera against heat-killed bacteria. These activities included Shwartzman skin reactivity, pyrogenicity, and animal lethality; however, the non-specific, resistance-enhancing effect of the endotoxins was not affected. Hyperimmune serum was later shown to contain immunoglobulins specific for the toxic moiety of the endotoxin (Radvany, Neale & Nowotny, 1966). Similar effects of anti-smooth endotoxin serum were reported by Tate, Douglas & Braude (1966), by Berczi (1967) and by Greisman, Young & DuBuy (1973). The last-mentioned study showed that the antisera neutralized the pyrogenicity of the homologous endotoxins but not that of heterologous preparations.

Emphasis has therefore been placed on developing antigens capable of eliciting cross-reactive, neutralizing antibodies, in an attempt to provide protection against a wide range of Enterobacteriaceae. Kunin (1963) described a common antigen in Enterobacteriaceae

and the antibody to this shared antigen opsonized heterologous Gram-negative bacilli for phagocytosis (Domingue & Neter, 1966). However, passively administered rabbit antisera to this common antigen failed to protect mice against Gram-negative bacterial infections (McCabe & Greely, 1973).

Another potential protective antigen of Enterobacteriaceae is the lipid moiety of lipopolysaccharide (LPS) which is believed to have a similar structure in different Gram-negative bacteria and to constitute the toxic site of the LPS (Lüderitz *et al.*, 1973). Accordingly, it might be expected that antiserum against the lipid A might neutralize endotoxins from a broad spectrum of Gram-negative bacteria and/or facilitate their removal by opsonization. Such antisera have been prepared by immunizing animals either against rough mutants or 'lipid A' preparations made by partial acid hydrolysis of LPS (Rank, Dipaul & Flügge-Rank, 1972; Galanos, Lüderitz & Westphal, 1971; Rietschel *et al.*, 1973).

Studies by Chedid *et al.* (1968) showed that horse hyperimmune serum against rough *Salmonella typhimurium* protected mice against infections with pathogenic *Klebsiella pneumoniae*. McCabe (1972) also reported that active and passive immunization with rough Rd_2 and Re mutants of *S. minnesota* offered significant protection against challenge with *K. pneumoniae* and *Escherichia coli*. More recently, Ziegler *et al.* (1973) showed that antiserum against a UDP-Gal epimerase-deficient mutant (Rc) of *E. coli* prevented lethal bacteraemia from pathogenic *E. coli* and *K. pneumoniae* in agranulocytic animals. All the above studies suggested the presence of common antigenic sites in the basal core of the LPS. More specifically, Galanos *et al.* (1971) prepared antibodies to 'lipid A' by using 'lipid A'-coated, acid-hydrolysed *S. minnesota* R595, and showed that anti-'lipid A' antibodies sensitized *E. coli* for intraperitoneal phagocytosis. However, recent studies by Mullan *et al.* (1974) have discounted any beneficial role of these antibodies in protection against Gram-negative infections in mice.

Our earlier *in vitro* studies (Ng *et al.*, 1974) showed that anti-*S. minnesota* R595 serum cross-reacted with *S. minnesota* R595 and *S. typhimurium* SL1102 Re glycolipids. The same serum neutralized the endotoxicity not only of these two glycolipids, but of LPS from smooth *S. minnesota* as well. In the present study, antisera were prepared against two Re mutants, *S. minnesota* R595 and *S. typhimurium* SL1102. The antisera were first tested for their serological cross-reactivities against various *E. coli* Re mutants and endotoxin preparations from other strains. In later experiments the antisera were tested against experimental infections with *K. pneumoniae* type II, *S. typhi* 0901, a bacteraemia isolate of *E. coli*, and *P. aeruginosa* 119. While the findings reported here indicate serological cross-reactivity between the Re mutants *in vitro*, passive transfer of such sera to normal animals did not protect them against lethal challenges with the above bacterial strains.

METHODS

Bacterial strains. Salmonella minnesota R595 Re mutant strain was obtained through the courtesy of Dr O. Lüderitz, Max Planck Institute, Freiburg, West Germany. The other Salmonella Re mutant, *S. typhimurium* SL1102, was obtained from Dr B. A. D. Stocker, Stanford University, California, U.S.A. *Escherichia coli* Re mutant, *E. coli* F515, was obtained from Dr G. Schmidt of the Max Planck Institute. Other *E. coli* mutants, *E. coli* D31m4 and D21f2, were gifts from Dr H. Boman of the University of Umea, Umea, Sweden. The bacteria were cultivated in a broth culture medium on a large scale in a fermenter, as previously described for *S. minnesota* R595 (Kasai & Nowotny, 1967).

The four Gram-negative bacteria used for challenge in mouse protection tests were

Pseudomonia aeruginosa 119, an isolate from a cystic fibrosis patient (obtained from Dr K. Cundy, Temple University School of Medicine), an *E. coli* isolate from a blood culture of a patient with bacteraemia (donated by Mrs K. Dietz, Temple University School of Medicine), *Salmonella typhi* 0901, an organism routinely used in our laboratories for non-specific resistance experiments, and *Klebsiella pneumoniae* type II (a gift from Dr L. Chedid, Institut Pasteur, Paris, France).

Isolation of glycolipids. Endotoxic glycolipids of the mutants were prepared as described previously (Ng *et al.*, 1974) by direct extraction of lyophilized cells with chloroform–methanol (4:1, v/v). The crude preparations were purified by precipitation with chloroform–methanol (1:2, v/v). Glycolipids were also prepared from *S. minnesota* R595 cells by the phenol–water extraction method (Lüderitz *et al.*, 1966) and the phenol–chloroform–petroleum ether procedure (Galanos, Lüderitz & Westphal, 1969).

Preparation of RESI from bacterial lipopolysaccharides. Endotoxic lipopolysaccharides were isolated from lyophilized cells of *Serratia marcescens*, *E. coli* 0127 and *P. fluorescens*, using the trichloroacetic acid extraction procedure of Boivin, Mesrobeanu & Mesrobeanu (1933). The preparations were further purified by the phenol–water procedure, and the products were precipitated by methanol and redissolved and sedimented by ultracentrifugation, as described previously (Nowotny *et al.*, 1966). 'Lipid A' precipitate was obtained from the lipopolysaccharides according to the procedure of Westphal & Lüderitz (1954). The LPS was hydrolysed with 1 M-HCl on a boiling water bath for 30 min. The 'lipid A' precipitate resulting was centrifuged and washed with water. The outlines of the procedure of Chang & Nowotny (1975) were followed to prepare RESI from the 'lipid A'. The lyophilized precipitate was extracted first with ethyl acetate containing 0·1 % acetic acid, and then with chloroform. The insoluble residue was called RESI. For the gel diffusion test, it was suspended in distilled water containing 0·5 % triethylamine.

Immunization. Antisera against *S. minnesota* R595 and *S. typhimurium* SL1102 heat-killed cells were prepared as described previously (Ng *et al.*, 1974). In the preparation of antisera against purified glycolipids from *S. minnesota* R595 cells, the chloroform–methanol (4:1)-soluble glycolipids were coated on autologous erythrocytes of New Zealand white rabbits. For the coating procedure, the same method was applied as described earlier for coating erythrocytes for passive haemagglutination, with the following minor modifications. About 10 ml of blood was drawn from each rabbit 2 days before immunization. It was divided into two 5 ml portions, each of which was mixed with 0·7 ml acid–citrate–dextrose anticoagulant solution and stored at cold-room temperature. On the day before immunization, the preserved blood was washed with saline (0·9 % NaCl) three times. After the final wash, the blood cells were centrifuged and 5 ml of *S. minnesota* R595 glycolipid suspension was added to the packed erythrocyte sediment. The chloroform–methanol (4:1)-soluble glycolipid suspension (1 mg ml^{-1}), prepared by sonicating the glycolipid in saline, was pre-heated in a boiling water bath for 2 h. The cell suspension and the cooled glycolipid were incubated under constant rotation at 37 °C for 1 h and overnight at 4 °C. The mixture was then washed twice with saline and the coating of glycolipid on red cells was verified by the passive haemagglutination test using antiserum against *S. minnesota* R595 of known titre. Finally, the coated red cells were sedimented and resuspended in 4 vol. saline. This suspension was used for injection for three consecutive days. Since six injections were given altogether, a new batch of red blood cells was prepared the day before the second series of immunization injections. Each rabbit was injected with 0·5 ml of glycolipid-coated red blood cell suspension on days 1, 2, 3, 8, 9 and 10. All animals were exsanguinated 21 days after the first injection.

Table 1. *Antibody titres of antisera prepared against S. minnesota* R595 *and S. typhimurium* SL1102 *cells and S. minnesota* R595 *glycolipid*

Antisera against:	Reciprocal titre*	
	S. minnesota R595 glycolipid	*S. typhimurium* SL1102 glycolipid
S. minnesota R595		
Rabbit No. 1	256	64
Rabbit No. 2	128	128
Rabbit No. 3	128	64
S. typhimurium SL1102		
Rabbit No. 1	64	64
Rabbit No. 2	128	256
Rabbit No. 3	256	128
S. minnesota R595 glycolipid coated on autologous erythrocytes	32	8

* Measured by the indirect haemagglutination method on microtitration plates. The glycolipid antigens were coated on sheep red blood cells.

Serological procedures. Passive haemagglutination, passive haemagglutination inhibition and double gel diffusion were performed as previously described (Nowotny, 1969).

Mouse protection tests. The challenging bacteria [*K. pneumoniae* type II, *S. typhi* 0901, *P. aeruginosa* 119 and *E. coli* (isolated from a bacteraemia patient)] were maintained on Trypticase soy agar slants. Overnight cultures of the organisms in brain–heart infusion broth were centrifuged and washed three times in saline (0·9 % NaCl) and resuspended again in saline. Serial dilutions were made from this suspension and viable counts made on agar plates. Female ICR mice (8 to 10 weeks old) were injected intravenously with 0·2 ml of culture and the LD_{50} determined as already described (Nowotny, 1969). In the passive protection experiments, the antiserum was given intravenously in a volume of 0·2 ml, 1 h before challenge with 1, 2 or 5 LD_{50} of bacteria.

Challenge of mice with opsonized bacteria. Bacterial suspensions prepared as described above were added to normal or immune rabbit serum. To 2 vol. of bacterial cell suspension were added 3 vol. of sterile immune or normal rabbit serum and the suspensions maintained at room temperature with constant rotation for 1 h. Before injection, the viable counts of bacterial cells in the suspensions were estimated, again by the agar plate count technique.

RESULTS

Immunization with bacteria and with Re glycolipid-coated autologous erythrocytes

We had previously found that isolated glycolipid preparations were non-immunogenic in rabbits, and also that antisera raised against whole Re mutant cells were not specific to the glycolipids of the cells only but contained antibodies against other cell wall components (unpublished data). Accordingly, rabbits were immunized with purified *S. minnesota* R595 glycolipid coated on autologous erythrocytes, as described above. As shown in Table 1, this new procedure gave rise to a low but significant antibody level.

Immunization with whole Re mutant cells of *S. minnesota* R595 and *S. typhimurium* SL1102 gave a somewhat higher titre to isolated glycolipid than immunization with glycolipid coated autologous erythrocytes. The antiserum titres determined by passive haemagglutination ranged from 64 to 256 (Table 1).

Table 2. *Cross-reactivity of antisera to Re cells and extracted glycolipid with glycolipids from various Re mutants*

Antisera prepared against:	Reciprocal titre to Re glycolipids*				
	S. minnesota R595	*S. typhimurium* SL1102	*E. coli* D31m4	*E. coli* D21f2	*E. coli* F515
S. minnesota R595	64	32	64	64	128
S. typhimurium SL1102	32	32	16	32	64
R595 glycolipid coated on autologous erythrocytes	32	8	16	64	32
S. minnesota R595 after absorption with R595 glycolipid†	0	0	0	0	0

* Measured by the indirect haemagglutination method on microtitration plates, as in Table 1.

† Antiserum was absorbed with an equal volume of glycolipid suspension (1 mg/ml) for 30 min at 37 °C followed by 1 h at 4 °C.

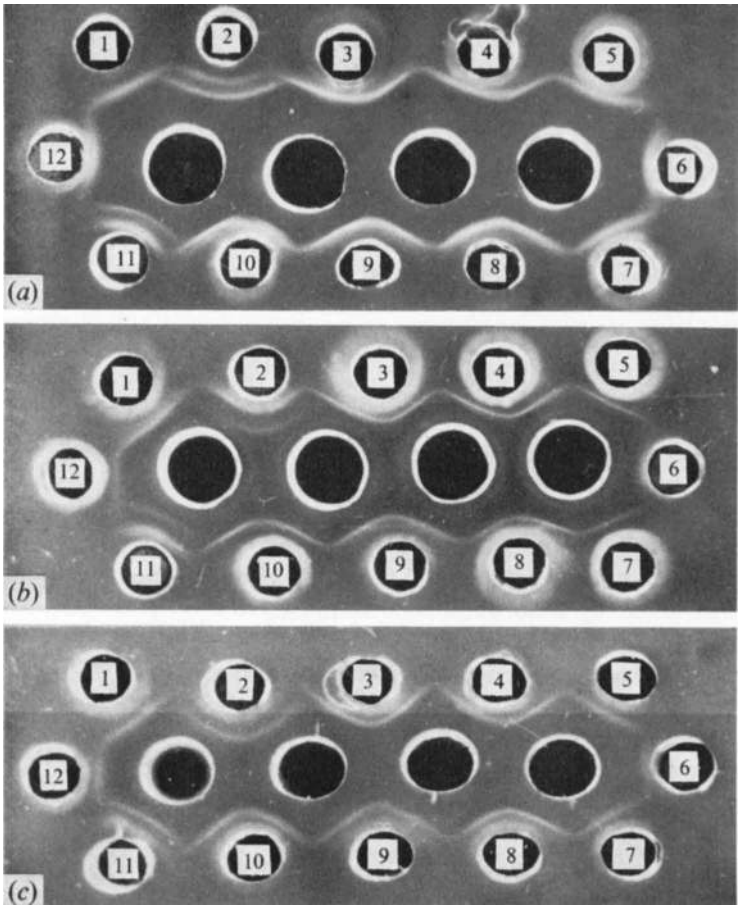

Fig. 1. Immunodiffusion test of various Re glycolipid preparations against antiserum prepared against Re mutants and Re glycolipid. (1), (9), *S. minnesota* R595 glycolipid; (2), (11), *S. typhimurium* SL1102 glycolipid; (3), (8), *E. coli* F515 glycolipid; (5), (7), (10), *E. coli* D21f2 glycolipid; (4), (6), (12), *E. coli* D31m4 glycolipid. Central wells contained anti-*S. minnesota* R595 in (*a*), anti-*S. typhimurium* SL1102 in (*b*) and anti-*S. minnesota* R595 glycolipid in (*c*).

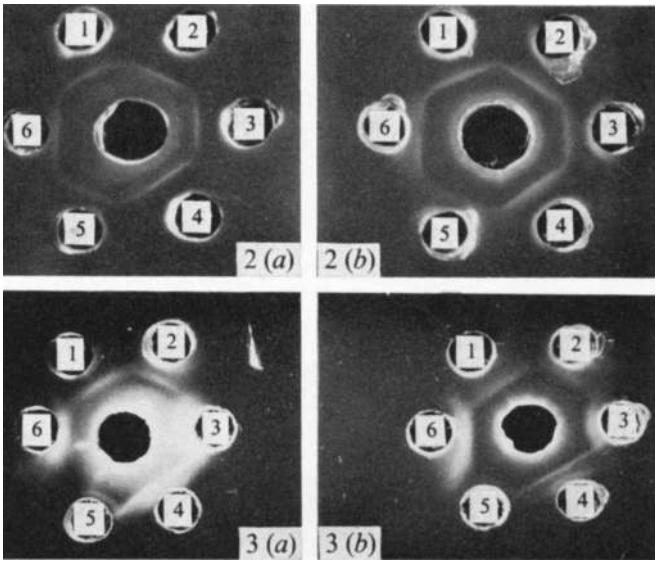

Fig. 2. Immunodiffusion test of Re glycolipids prepared by different methods against antisera prepared against Re mutants. (1) (2), *S. minnesota* R595 glycolipid, phenol–chloroform–petroleum ether method; (3) (4), *S. minnesota* R595 glycolipid, phenol–water method; (5), *S. typhimurium* SL1102 glycolipid, chloroform–methanol method; (6), *S. minnesota* R595 glycolipid, chloroform–methanol method. Central wells contained (*a*) anti-*S. minnesota* R595 and (*b*) anti-*S. typhimurium* SL1102.

Fig. 3. Immunodiffusion test of various 'lipid A' preparations against antisera prepared against Re mutants. (1), (2), *Ser. marcescens* RESI; (3), ethyl acetate extract of *Ser. marcescens* 'lipid A'; (4) *E. coli* 0127 RESI; (5), ethyl acetate extract of *E. coli* 0127 'lipid A'; (6), *P. fluorescens* RESI. Central well contained (*a*) anti-*S. typhimurium* SL1102 and (*b*) anti-*S. minnesota* R595.

Cross-reactivity of the antisera

In the passive haemagglutination test, shown in Table 1, it was also evident that the rabbit anti-Re cell sera cross-reacted with both *S. minnesota* R595 and *S. typhimurium* SL1102 glycolipids. In addition, the two antisera also cross-reacted with the glycolipids extracted from the three *E. coli* Re mutants, *E. coli* F515, *E. coli* D31m4 and *E. coli* D21f2, as demonstrated again by passive haemagglutination (Table 2). The specificity of the anti-*S. minnesota* R595 serum was tested by absorption with *S. minnesota* R595 glycolipid. This absorption completely removed antibody to all five glycolipids (Table 2).

The cross-reactivity among the various glycolipids was more conclusively demonstrated by gel diffusion. As shown in Fig. 1 (*a*) and (*b*) both the above antisera formed reactions of identity with glycolipids from all five Re mutants.

The two antisera were also tested against *S. minnesota* R595 endotoxins prepared by the three different extraction procedures described in Methods. Both anti-*S. minnesota* R595 and anti-*S. typhimurium* SL1102 cross-reacted with all three preparations, again giving lines sndicating antigenic identity (Fig. 2).

The antiserum prepared by injecting glycolipid-coated autologous erythrocytes, which is more specific than antisera obtained by immunization with whole Re cells, also cross-reacted with the three *E. coli* Re glycolipids. Table 2 shows the results of passive haemagglutination and Fig. 1 (*c*) shows the patterns of gel diffusion. In the latter, the precipitation lines again show antigenic identity.

Table 3. *Effect of antisera to Re cells and to isolated glycolipid on lethal bacterial infection in mice*

Pretreatment*	Survivors (%) following challenge†: *S. typhi* 0901 (2 LD_{50})	*E. coli* (5 LD_{50})	*P. aeruginosa* 119 (5 LD_{50})	*K. pneumoniae* type II (1 LD_{50})
Saline	0	0	10	30
Normal rabbit serum	0	0	0	50
Anti-*S. minnesota* R595	0	0	0	40
Anti-*S. typhimurium* SL1102	20	0	0	50
Anti-*S. minnesota* R595+ *S. typhimurium* SL1102‡	—	0	0	—
Anti-*S. minnesota* R595 glycolipid coated on autologous erythrocytes	—	0	0	40

* Mice in groups of ten were given 0·2 ml of saline or sera intravenously 1 h before challenge.
† Challenge organisms were introduced intravenously in 0·2 ml volume. Survival was estimated at 48 h in all cases except for *K. pneumoniae* type II where it was estimated at 4th day.
‡ The antisera were mixed in equal volume.

Table 4. *Survival of mice after intravenous challenge with P. aeruginosa* 119 *incubated in antiserum to Re cells and to S. minnesota* R595 *glycolipid*

Groups received *P. aeruginosa* 119 incubated with*:	72 h survivors (%) at challenge dose of: 1 LD_{50}	5 LD_{50}
Saline	30	0
Normal rabbit serum	50	0
Anti-*S. minnesota* R595	60	0
Anti-*S. typhimurium* SL1102	80	0
Anti-*S. minnesota* R595 glycolipid coated on autologous erythrocytes	30	0

* Bacterial suspension (0·2 ml) was incubated with 0·3 ml of saline or serum for 1 h at room temperature before injection. Ten mice per group were used.

In the gel diffusion test, the anti-*S. minnesota* R595 and *S. typhimurium* SL1102 antisera were also tested for their ability to cross-react with various RESI preparations. As shown in Fig. 3, both antisera cross-reacted with RESI preparations from LPS of *E. coli* 0127, *P. fluorescens* and *Serratia marcescens.*

Attempts at passive protection of mice with immune sera

As a result of the above observations that antisera against Re mutant cells, or Re glycolipid, cross-reacted with endotoxic components from various Gram-negative bacteria, tests were made to see if the antisera would protect mice against Gram-negative infections. Four strains of Gram-negative bacteria, *K. pneumoniae* type II, *S. typhi* 0901, *P. aeruginosa* 119 and an *E. coli* strain, were used for challenging passively immunized and untreated animals. The LD_{50} for the four bacteria was determined as $1{\cdot}5 \times 10^3$, $5{\cdot}0 \times 10^8$, $1{\cdot}0 \times 10^7$ and $8{\cdot}0 \times 10^7$ cells, respectively. No protective effect of anti-*S. minnesota* R595 (titre 256) and anti-*S. typhimurium* SL1102 (titre 256) sera in mice challenged with 1, 2 or 5 LD_{50} of the challenge bacteria was observed (Table 3).

Injection of antibody-coated bacteria into mice

The above antisera were used to pre-coat the bacterial challenge suspensions before injection into mice. The results in Table 4 compare survival of mice challenged with bacteria incubated either in antiserum, in normal serum or in saline. In these experiments, *P. aeruginosa* 119 was used and all three antisera described above were added to either 1 or 5 LD_{50} challenge doses. Using the 1 LD_{50} challenge dose, anti-*S. typhimurium* SL1102 seemed to increase the survival rate of animals slightly, although the results are not statistically significant. Anti-*S. minnesota* R595 and anti-glycolipid (on autologous erythrocyte) antisera showed similar results to normal serum. When the challenge dose was increased to 5 LD_{50}, all experimental animals died.

DISCUSSION

While isolated glycolipid injected into rabbits showed no detectable antibody production the same material proved to be immunogenic when coated on rabbit autologous red blood cells (Tables 1 and 2, Fig. 1*c*). This indicates that at least some of the immunodeterminants present in a glycolipid molecule are sufficiently exposed to manifest immunogenicity when coated on to red blood cells. This antiserum, as compared with antisera elicited by the injection of glycolipid-containing whole cells, is specific for the glycolipid molecule only, since nothing else foreign has been injected. Such antiserum may find several uses, such as in the investigation of the exposure of the lipid moiety of LPS on bacterial surfaces, or in studying the role of antibodies specific against the lipid moiety in host resistance against infection, as will be discussed later. The immunization procedure developed may also be generally applicable in raising specific antibody to any antigenic but not immunogenic compounds that bind to autologous erythrocytes of animals used for immunization.

The aim of the studies reported here was twofold. First, we wished to investigate the existence of cross-reacting immunodeterminants in a variety of Gram-negative bacterial endotoxins. Secondly, and more important, we wished to see what role immunoglobulins, directed against certain immunodeterminants present in the lipid moiety of Gram-negative endotoxins, may have in host resistance against Gram-negative infections.

In a previous study (Ng *et al.*, 1974), we used the passive haemagglutination and Ouchterlony tests to demonstrate cross-reactivity and immunological identity between Re glycolipids from *S. minnesota* R595 and *S. typhimurium* SL1102. An earlier report by Schmidt, Jann & Jann (1970) showed cross-reactivity between the Re glycolipids from *S. minnesota* R595 and *E. coli* F515 by the passive haemagglutination method. In the present study we found serological cross-reactivity among the above two Salmonella Re glycolipids and three Re glycolipids from *E. coli* F515, *E. coli* D31m4 and *E. coli* D21f2 by the same methods (Tables 1 and 2, Fig. 1). The antisera which cross-reacted with the five Re glycolipids were anti-*S. minnesota* R595 and anti-*S. typhimurium* SL1102 sera as well as antiserum prepared against purified *S. minnesota* R595 glycolipid coated on autologous red blood cells of rabbits. As Fig. 1 shows, one major gel diffusion precipitin band is shared by all preparations tested. On the other hand, certain antisera formed additional precipitin bands with a few glycolipid preparations. This seems to indicate that not all immunodeterminants present in these glycolipids are equally shared by all preparations tested.

The results discussed above were obtained by using glycolipids extracted from heptoseless Re mutants of Gram-negative bacteria which synthesize only the lipid moiety of endotoxic lipopolysaccharide of parental strains. Chang & Nowotny (1975), attempted to obtain

partially degraded fractions of smooth endotoxic lipopolysaccharide, devoid of polysaccharide but still maintaining characteristic endotoxic reactions. Such material was isolated from smooth lipopolysaccharides of *Ser. marcescens*, *E. coli* 0127 and *P. fluorescens*. The preparation, called RESI, was fully active in a number of endotoxicity parameters. In the present studies, cross-reactivity among the above three RESI preparations with anti-*S. minnesota* R595 and anti-*S. typhimurium* SL1102 was determined. The results (Fig. 3) showed that such RESI preparations, which seem to represent the most essential core of the active site of endotoxin molecules, also carry immunodeterminants present in Re glycolipid, synthesized by defective mutant strains. This indicates that the smooth LPS molecule may be reduced by chemical procedures to a greatly simplified and yet highly endotoxic fragment. At least one immunodeterminant present in this fragment seems to be present in a number of unrelated Gram-negative smooth or rough endotoxins.

The above findings, which showed that there is a cross-reactive antigenic site(s) in the lipid moiety of LPS, prompted us to investigate the possible beneficial role of antibodies directed at the lipid moiety of LPS in animal immunity to bacterial infections. Disappointingly enough, in the present studies repeated attempts to protect mice against Gram-negative bacterial infections with antisera against Re mutants such as *S. minnesota* R595 and *S. typhimurium* SL1102 and antiserum against purified *S. minnesota* R595 glycolipid failed (Tables 3 and 4).

The discrepancy observed in our results and those of others (Chedid *et al.*, 1968; McCabe, 1972; Ziegler *et al.*, 1973) can be partially explained by the fact that different animal strains, different challenge organisms and different antisera preparations were used. For example, Chedid *et al.* (1968) used hyperimmune horse serum to *S. typhimurium* to protect mice against *K. pneumoniae* type II. Horses are better immunoglobulin producers to polysaccharide antigens than are rabbits. Furthermore, his antiserum has been produced by the injection of Ra and not Rc mutants. We cannot explain the differences between our findings and those reported earlier (McCabe, 1972), where an identical strain was used as the challenge organism and the preparation of the antisera as well as their Re glycolipid antibody titre were quite comparable. Recently Mullan *et al.* (1974) also found no significant protection in mice against infection with *K. pneumoniae* and *E. coli* with passive administration of antiserum prepared against 'lipid A' of *S. minnesota* R595. Our findings and conclusions are in agreement with the report of Mullan *et al.* (1974). The results of our serological studies clearly demonstrate that there are cross-reactive and/or identical immunodeterminant groups in the lipid moiety of LPS. Antibody to these may neutralize the endotoxicity of isolated endotoxins, but may not be able to reach the cross-reactive determinants present in the lipid moiety of living bacteria.

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