INFECTION AND IMMUNITY, Jan. 1977, p. 34-49
Copyright © 1977 American Society for Microbiology

Vol. 15, No. 1
Printed in U.S.A.

Lipid A Antiserum-Mediated Protection Against Lipopolysaccharide- and Lipid A-Induced Fever and Skin Necrosis

ERNST Th. RIETSCHEL* AND CHRIS GALANOS

Max-Planck-Institut für Immunbiologie, D-78 Freiburg, West Germany

Received for publication 19 July 1976

The antiendotoxic activity of lipid A antiserum was studied in rabbits, using lipid A (lipopolysaccharide)-induced fever and skin necrosis as test systems. It was found that lipid A antiserum had no significant antipyretic effect when it was incubated with lipid A or injected intravenously before lipid A challenge. However, in animals that were pretreated (day 0) with a single dose of lipid A (lipopolysaccharide), a significant protective effect of passively transferred antiserum (day 1) to lipid A (lipopolysaccharide fever) (day 2) was observed. Also, the lipid A (lipopolysaccharide)-induced local Shwartzman reaction could be prevented by lipid A antiserum. In the fever system, the degree of protection depended on the preparative and the challenge doses as well as on the amount of antiserum transferred. The fever protection mediated by lipid A antiserum seemed to be lipid A (lipopolysaccharide) specific with regard to both the preparative and the challenge injections. Lipid A specificity of the protective factor present in the antiserum was indicated by the fact that it could be absorbed from the serum with lipid A. Preliminary experiments suggest that the factor might be identical with lipid A-specific immunoglobulin. The significance of the preparative injection is not understood at the present time. It is concluded, however, that in the fever protection system described, besides specific humoral factors, other factors, perhaps cellular, are involved.

Bacterial lipopolysaccharides are known to be potent toxins (endotoxins). Their injection into higher organisms leads to the development of a variety of pathophysiological effects including fever, leucopenia, leucocytosis, the Shwartzman reaction, and, in larger doses, shock and death. Chemically, lipopolysaccharides consist of a heteropolysaccharide (O-specific chain, core) and a covalently bound lipoidal component (lipid A). The O-specific chain determines the serological specificity, whereas the lipid A component represents the toxic principle of the molecule (16, 17).

The possible role of endotoxins in gram-negative sepsis (intravascular coagulation, irreversible shock) has prompted studies aimed at an immunological control of endotoxicity. Earlier workers showed that serum from endotoxin-immunized animals conferred passive protection against endotoxin effects such as lethality, pyrogenicity, and Shwartzman reactivity (for summary, see reference 9). Greisman et al. (10), using the fever test, found that protection was maximal with sera raised against the homologous S-form lipopolysaccharides and that it was mediated by O-specific antibodies (immunoglobulins M and G). (O specificity of passive protection was also found by others [see reference 9].) However, when larger amounts of serum were transferred and with antisera against R-forms, a certain degree of cross-protection to lipopolysaccharides with different O specificities was observed (10). Such a heterologous protection has also been described by Braude et al. (2, 3), who used the local and generalized Shwartzman reaction as a test system. These findings suggested that cross-protection was mediated by antibodies directed against determinants shared by lipopolysaccharides of different O specificities. Such common structures are present in the lipopolysaccharide core and also in the toxic lipid A component, which, according to chemical studies, is structurally similar in various bacterial groups (11).

The existence of protective lipid A antibodies has been postulated by Kim and Watson (13-15), but only recently have the preparation and quantitation of lipid A-specific antisera been described (6). At this time lipid A antibodies were observed to cross-react with lipid A of lipopolysaccharides from various bacteria. The availability of lipid A antisera allowed investi-

gations on their potential protective power against endotoxicity.

In the present paper, the possible antiendotoxic activity of lipid A antiserum on lipid A (lipopolysaccharide)-induced fever and skin necrosis (local Shwartzman reaction) was analyzed in rabbits. It will be shown that lipid A antiserum, after passive transfer, confers significant protection against skin necrosis and against fever, provided the test animals have been pretreated with endotoxin.

(Parts of the results were presented at the International Workshop on Immunological and Biological Properties of Peptidoglycan and Related Bacterial Cell Wall Polymers, Munich, 1974 [23] at the Pharmacology Meeting, Graz, 1974 [20], and at the American Society for Microbiology Conference on Pathogenic Mechanisms in Bacterial Diseases, New Orleans, 1975 [21].)

MATERIALS AND METHODS

Animals. For pyrogen assays, female and male hybrid rabbits (white Vienna × Alaska), weighing between 1.7 and 2.2 kg and bred under specific-pathogen-free conditions, were used. The local Shwartzman reaction was produced in white New Zealand rabbits weighing approximately 2 kg. The animals were housed in an air-conditioned room, in which biological tests were performed.

Pyrogens. S-form lipopolysaccharides from *Salmonella typhi* and *S. abortus-equi* were prepared by phenol-water extraction (26), and R-form lipopolysaccharides from *Salmonella minnesota* (Re), *Shigella flexneri* 5b (Re), *Escherichia coli* EH100 (Ra), and *E. coli* (Re) were prepared by the phenol-chloroform-petroleum ether method (5). The origins of the corresponding bacteria have been described (7, 11).

Lipid A was isolated from lipopolysaccharides of *S. minnesota* R60 (Ra) and *S. minnesota* R595 (Re) by mild acid hydrolysis (7). It was shown to be free of KDO and heptose. Lipid A/bovine serum albumin (BSA) complexes contained lipid A and pyrogen-free BSA in a ratio of 1:1 (wt/wt) (7).

Lipid A vaccine (acid-treated *S. minnesota* Re coated with additional lipid A in a ratio of 10:1 [wt/wt]) was prepared as described earlier (6).

Viral double-stranded ribonucleic acid (dsRNA) was a gift of Janet Dewdney (Beecham Research Laboratories, England). The pyrogenicities (MPD-3 values) of the different pyrogens used in this study are summarized in Table 1.

Stock solutions of pyrogens in pyrogen-free, deionized water were kept at 4°C and diluted with phosphate-buffered saline (pH 7, 0.15 M) before the experiment. Buffers, deionized water, glassware, syringes, and other equipment used in this study were sterile and pyrogen free.

Antisera. Lipid A antiserum was prepared by intravenously (i.v.) immunizing 10 rabbits with increasing doses (in micrograms per kilogram) of lipid A vaccine as follows: 100 (day 0), 200 (day 7), 400 (day 14), and 800 (day 21). These animals were tolerant (fever test) to 100 MPD-3 of the vaccine or lipid A/BSA per kg when tested on day 23. The animals were bled on days 28 and 35 by cardiac puncture, and the sera obtained were pooled. Three pools of antisera were prepared, all exhibiting similar serological anti-lipid A activity (reciprocal hemolytic titer, 2,000 to 4,000). In sera of all normal (untreated) rabbits, no anti-lipid A hemolytic activity could be detected. All antisera used in this study were found to be nonpyrogenic.

TABLE 1. *Pyrogenicity (MPD-3) of pyrogens used*

Pyrogen	Pyrogenicity (MPD-3) (μg)
Salmonella abortus-equi (S) LPS[a]	0.002
S. typhi (S) LPS	0.005
Chromabacterium violaceum (S) LPS	0.002
S. minnesota (Re) LPS	0.005
Shigella flexneri (Re) LPS	0.005
Escherichia coli (Re) LPS	0.005
Lipid A/BSA (*S. minnesota*)	0.008
Lipid A vaccine	1.0
dsRNA	0.01

[a] LPS, Lipopolysaccharide.

Goat lipid A antiserum was prepared by immunizing a goat with three doses (in milligrams) of lipid A vaccine as follows: 2 (day 0), 5 (day 60), and 5 (day 360). For each immunization, the vaccine was administered in incomplete Freund adjuvant and divided into subdoses, which were injected into four different parts of the body. Two weeks after the last injection, the animal was exsanguinated under anesthesia. The serum obtained was precipitated with ammonium sulfate (2 M, final concentration), the precipitate was dialyzed against phosphate-buffered saline, and the volume was adjusted to that of the starting serum. Its hemolytic anti-lipid A activity (reciprocal titer) was 8,000. BSA antiserum (1 mg of anti-BSA antibody/ml) and antiserum raised against cross-linked polysaccharide from *S. typhi* lipopolysaccharide were gifts of H. Mossmann and W. Falk of this institute.

Antibody determinations. Anti-lipid A activity of lipid A antisera was determined by passive hemolysis, using erythrocytes (human B, Rh^+) coated with alkali-treated lipid A in the presence of complement, and is expressed as hemolytic titers (6). Anti-*S. typhi* activity was tested similarly (passive hemolysis) with erythrocytes sensitized by alkali-treated *S. typhi* lipopolysaccharide. The sera analyzed for anti-*S. typhi* activity had been previously absorbed with lipid A (see below).

Absorption of lipid A antiserum with lipid A. Absorption of lipid A antiserum with lipid A has been described (6). Briefly, formalinized erythrocytes (0.1 ml of sediment, human B, Rh^+) were coated with alkali-treated lipid A (0.5 mg, 1 h, 37°C) and washed five times with water. The coated erythrocytes were mixed with lipid A antiserum (1 ml;

titer, 1:2,048) and kept for 1 h at 4°C. The absorbed serum, obtained after centrifugation, exhibited no detectable anti-lipid A activity (hemolytic titer < 1:4) and was not pyrogenic.

Pyrogen assay. Pyrogen assays in rabbits were performed in an air-conditioned room as described (25). At least four animals were used per test. Fever responses were measured rectally using thermistor probes connected to a recording temperature-measuring device (Hartmann und Braun, Frankfurt). Pyrogenicity was quantitated by determining the minimal pyrogenic dose causing a 0.6°C rise in temperature 3 h after i.v. injection (MPD-3), as described previously (22, 25). In most experiments, a dose corresponding to 100 MPD-3 of pyrogen per kg was used. This dose is known to induce a biphasic fever response in normal rabbits, the maximal fever (second fever peak) occurring 3 h after i.v. injection of the pyrogen (Fig. 1A). Therefore, in the figures of this paper, the change in temperature after 3 h (ΔT-3) is given as a measure of fever or protection against fever. Figure 1B shows a typical (monophasic) fever response to 100 MPD-3 of pyrogen per kg in protected (tolerant) rabbits (25).

Local Shwartzman reaction (2). New Zealand white rabbits (2 kg) were prepared with lipopolysaccharide (40 μg in 0.25 ml of phosphate-buffered saline) at four different skin sites. The reaction was provoked 24 h after preparation by i.v. injection of either lipid A/BSA (40 μg) or lipopolysaccharide (*S. abortus-equi*, 10 μg; *S. typhi*, 20 μg). Hemorrhage or necrosis of the skin after provocation was recorded as a positive reaction.

Statistical methods. Student's *t* test was used to determine whether the effect of incubation of lipid A/BSA with lipid A antiserum (as compared with that of incubation with normal rabbit serum [NRS]) or whether the effect of lipid A-absorbed antiserum (as compared with that of NRS or nonabsorbed antiserum) was significant.

RESULTS

Incubation of lipid A/BSA with lipid A antiserum. Lipid A/BSA (0.8 μg = 100 MPD-3) was incubated (37°C, 1 h) with lipid A antiserum (1 and 7.5 ml). Lipid A/BSA alone and lipid A/BSA (0.8 μg) treated with 7.5 ml of NRS served as controls. The incubation mixture was then injected i.v. into groups of rabbits. Preincubation with NRS had no effect on the pyrogenicity of lipid A/BSA (ΔT-3 = 1.8 ± 0.3°C) (Fig. 2). (Fever response of lipid A/BSA alone: ΔT-3 = 1.9 ± 0.3°C.) After incubation with antiserum, pyrogenicity was partly reduced, but not significantly (1 ml: $P < 0.1$; 7.5 ml: $P < 0.1$). It is clear from Fig. 2 that the pyrogenicity of lipid A/BSA was not or was only slightly effected by preincubation with either NRS or lipid A antiserum.

Passive transfer of lipid A antiserum. Groups of rabbits received lipid A antiserum i.v. (7.5 ml/kg), simultaneously with or 1, 5, 24, and 48 h before a challenge with lipid A/BSA (0.8 μg/kg). In no case was the 3-h fever response significantly different from that of control animals injected i.v. with NRS (7.5 ml/kg) 5 h before challenge (Fig. 3, lower panel). Anti-lipid A antibody titers were determined just before lipid A/BSA injection in animals that had received the antiserum 24 and 48 h before challenge. In both groups, hemolytic titers were on the order of 1:200. Thus, despite the presence of circulating lipid A antibodies, rabbits passively immunized with lipid A antiserum were not protected against lipid A pyrogenicity.

Passive transfer of lipid A antiserum in lipid A/BSA-pretreated rabbits. In another series of experiments, the possible protective effect of lipid A antiserum was examined in lipid A-pretreated animals. Rabbits were given 100 MPD-3 of lipid A/BSA (i.v.) per kg on day 0. A normal fever response was observed (ΔT-3 = 1.9 ± 0.2°C). After 24 h (day 1), the animals received lipid A antiserum (0.25 ml/kg, i.v.). After another 24 h (day 2), the animals were

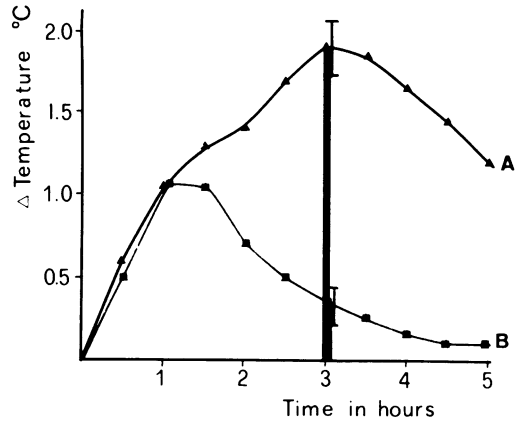

FIG. 1. *Typical fever response to a 100 MPD-3 dose (i.v.) of lipid A/BSA of normal rabbits (A) and rabbits made tolerant to lipid A (B) (22, 25).*

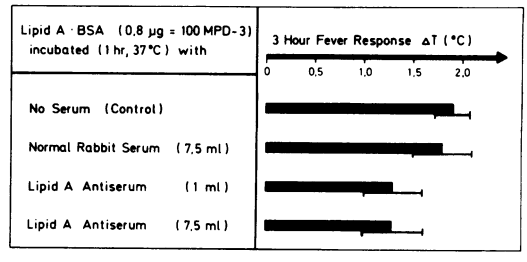

FIG. 2. *Effect of incubation of lipid A antiserum with lipid A/BSA on lipid A/BSA pyrogenicity. Shown are the fever responses (ΔT-3) in groups of rabbits to lipid A/BSA (100 MPD-3/kg, i.v.) previously incubated with NRS (7.5 ml/kg) or lipid A antiserum (1 and 7.5 ml/kg).*

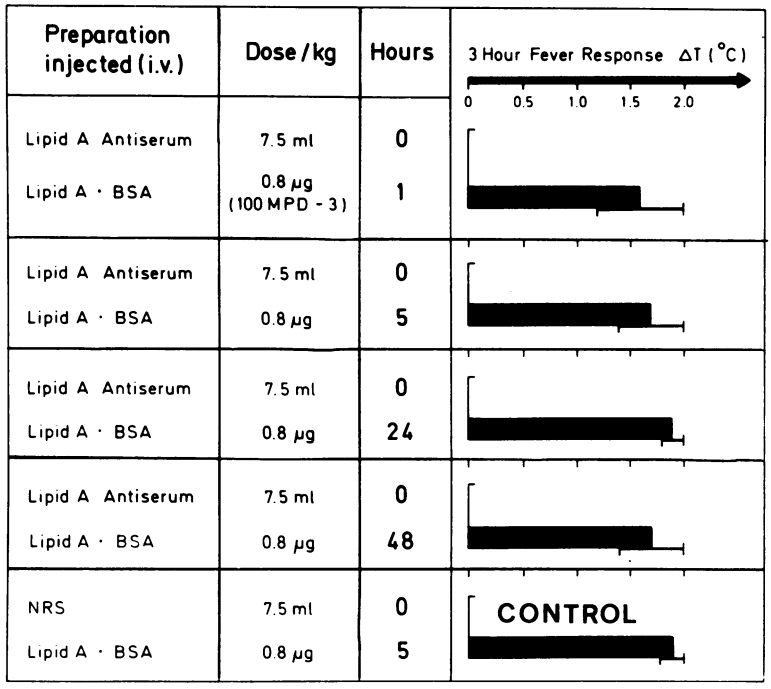

FIG. 3. *Effect of passively transferred lipid A antiserum on lipid A/BSA pyrogenicity. Shown are the fever responses (ΔT-3) to lipid A/BSA (100 MPD-3/kg, i.v.) in groups of rabbits preinjected (i.v.) with lipid A antiserum (7.5 ml/kg) or NRS (7.5 ml/kg, lower panel) at the times indicated.*

challenged with 100 MPD-3 of lipid A/BSA (i.v.) per kg. The animals treated in this way did not respond with fever to this second, otherwise pyrogenic dose (ΔT-3 = 0.2 ± 0.1°C) (Fig. 4A). That this protective effect was caused specifically by the lipid A antiserum was demonstrated in control experiments where no serum, NRS, or BSA antiserum was given under otherwise identical conditions. In each case a normal (biphasic) fever response was seen (Fig. 4B–D).

It was found that as little as 0.25 ml of lipid A antiserum per kg was sufficient to afford significant protection. Higher amounts of antiserum (up to 7.5 ml/kg) also gave good protection, whereas lower amounts (0.1 ml/kg) were not effective.

Rabbits that had been given lipid A/BSA (100 MPD-3/kg, day 0) and lipid A antiserum (0.25 ml/kg, day 1) did not show any detectable hemolytic anti-lipid A activity in their sera on day 2 before challenge.

It should also be mentioned that lipid A antiserum from other species conferred protection to lipid A fever in rabbits. Lipid A antiserum from goats was as effective as antiserum from rabbits (ΔT-3 = 0.6 ± 0.3°C). Also, lipid A antiserum from vervets was found to be protective in rabbits (L. Helm, U. Hägele, and O. Westphal, personal communication).

These results show that lipid A antiserum exhibits a protective effect on lipid A/BSA-induced fever, provided the test animals have been pretreated with lipid A/BSA.

Time of lipid A antiserum transfer. In the previous experiment (Fig. 4A), lipid A antiserum had been transferred on day 1, i.e., between the preparative (day 0) and challenge (day 2) injections of lipid A/BSA. To gain information on the optimal time of transfer, lipid A antiserum was given to groups of rabbits simultaneously with the preparative injection of lipid A/BSA (day 0, Fig. 5A) or with the lipid A/BSA challenge (day 2, Fig. 5D) as well as at times between the two pyrogen injections (Fig. 5B, C). In all cases, a similar degree of protection was observed. However, when lipid A antiserum was given 1 h after the lipid A/BSA challenge (day 2, Fig. 5E), an almost normal biphasic fever response was observed.

Thus, no difference with regard to the degree of protection was seen when the lipid A antiserum was given simultaneously with or between the preparative or the challenge dose of lipid A. In some of the following experiments the antiserum was transferred on day 1, and in others 1 h before challenge.

Duration of the effect of lipid A/BSA pretreatment. Rabbits were pretreated with 100

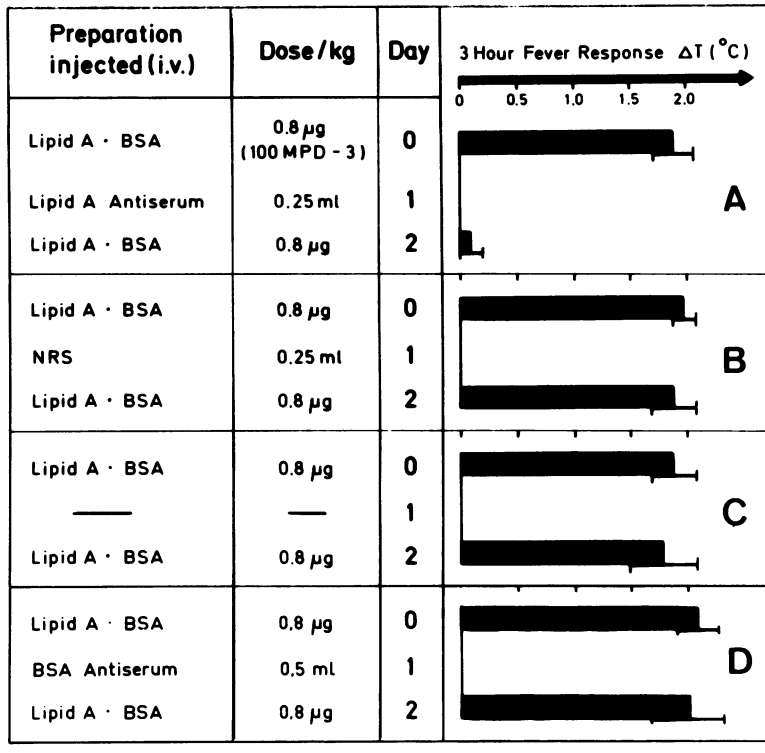

FIG. 4. *Tolerance to lipid A/BSA fever induced by lipid A antiserum in lipid A/BSA-pretreated rabbits. Shown are the fever responses (ΔT-3) to lipid A/BSA (day 2, i.v.) in groups of rabbits pretreated (i.v.) on day 0 with lipid A/BSA and on day 1 with lipid A antiserum (A), NRS (B), no serum (C), or BSA antiserum (D). Doses of lipid A/BSA and amounts of sera transferred are as indicated on the figure.*

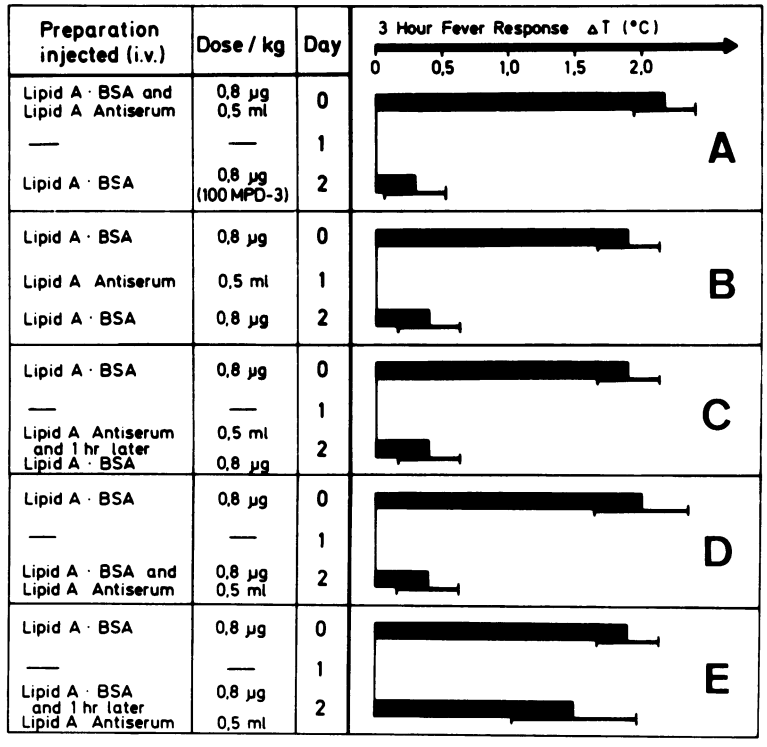

FIG. 5. *Determination of optimal time of lipid A antiserum transfer to induce fever tolerance in lipid A/BSA-pretreated rabbits. Shown are the fever responses (ΔT-3) to lipid A/BSA challenge (day 2, i.v.) in groups of lipid A/BSA-pretreated (day 0, i.v.) rabbits to which lipid A antiserum was given (i.v.) simultaneously with pretreatment (A), between pretreatment and challenge (B, C), simultaneously with the challenge (D), and 1 h after the challenge (E). Doses of lipid A/BSA and amounts of antiserum transferred are as indicated in the figure.*

MPD-3 of lipid A/BSA per kg on day 0. Groups of these animals were given lipid A antiserum (0.5 ml/kg) on days 2 through 7. Each group was then challenged with 100 MPD-3 of lipid A/BSA per kg 1 h after antiserum transfer (Fig. 6). A protective effect of the antiserum, although decreasing, could be observed in those animals that had been pretreated with lipid A 2, 3, 4, or 5 days before challenge. When the time between pretreatment and challenge was extended to 6 or 7 days, the antiserum did not express a protective activity. Thus, the effect of pretreatment with 100 MPD-3 of lipid A/BSA per kg lasts approximately 5 days.

Dose dependency of protection. The dependence of the degree of protection on the preparative lipid A/BSA dose (day 0) is shown in Fig. 7. In rabbits pretreated with only 1 MPD-3 of lipid A/BSA per kg (day 0) and lipid A antiserum (0.25 ml/kg, day 1), no significant protection was observed on challenge (day 2) with 100 MPD-3 of lipid A/BSA per kg (Fig. 7A). Pretreatment of rabbits with 10 MPD-3 of lipid A/BSA per kg led to partial protection (Fig. 7B), whereas injection of 100 MPD-3 of lipid A/BSA per kg gave maximal protection (Fig. 7C, 4A, and 5A–D). Lipid A antiserum-mediated resistance to fever was also dependent on the challenge dose (Fig. 8). When rabbits pretreated with 100 MPD-3 of lipid A per kg (day 0) and lipid A antiserum (0.25 ml/kg, day 1) were challenged on day 2 with 1,000 MPD-3 (8 μg) of lipid

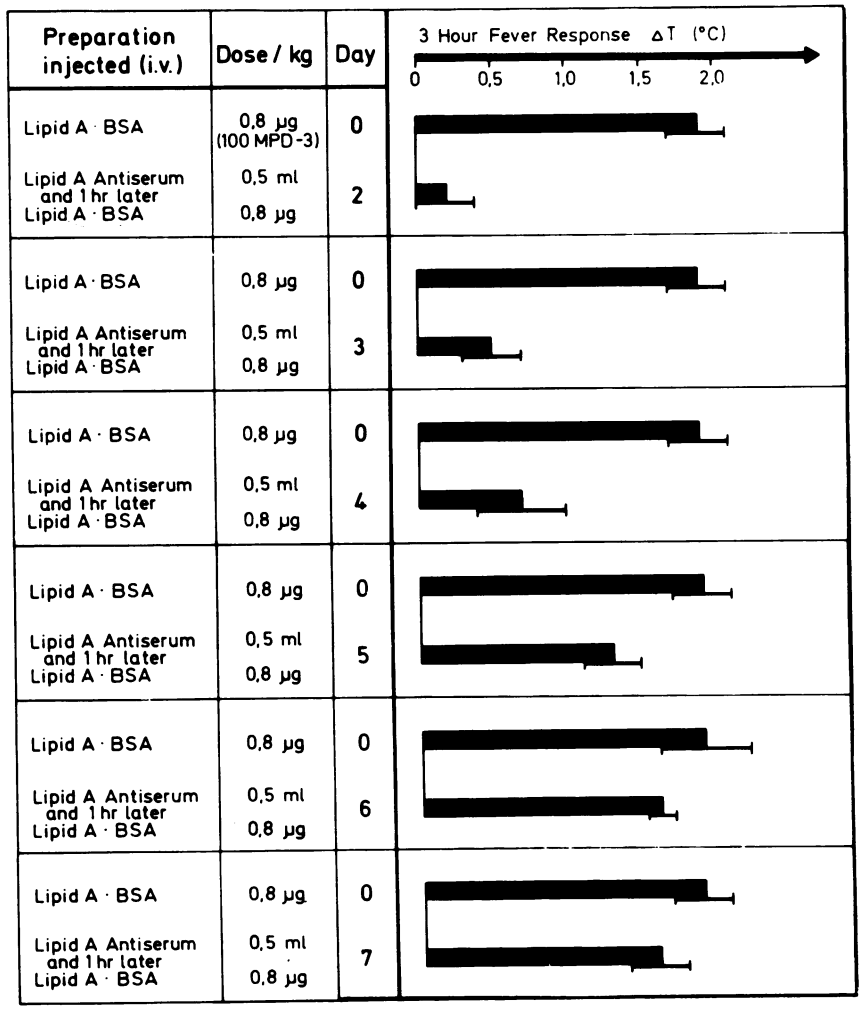

FIG. 6. *Duration of effect of lipid A/BSA pretreatment on lipid A antiserum-mediated fever tolerance. Shown are the fever responses (ΔT-3) in groups of rabbits pretreated with lipid A/BSA (day 0, i.v.) to a lipid A/BSA challenge (i.v.) on days 2 through 7. Lipid A antiserum was injected 1 h before challenge. Doses of lipid A/BSA used and amounts of antiserum transferred are as indicated in the figure.*

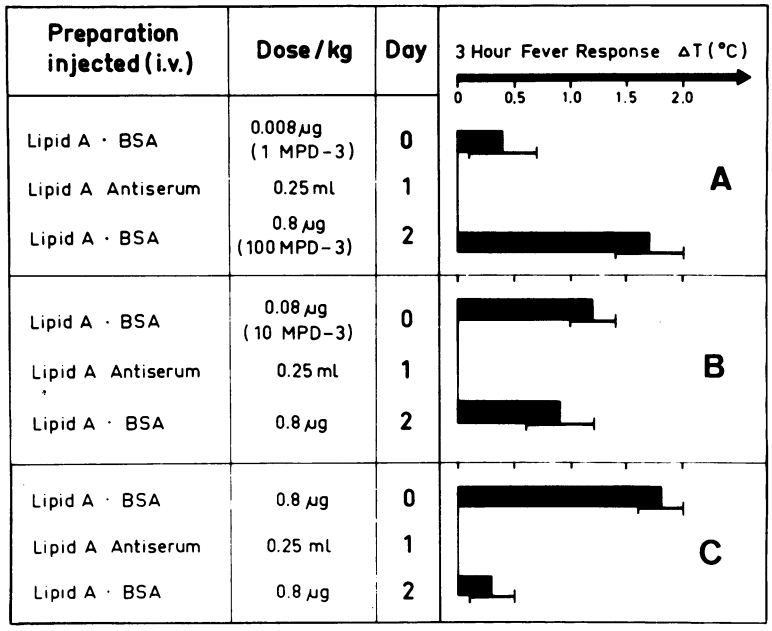

FIG. 7. *Dependency of the degree of lipid A antiserum-mediated fever tolerance on the pretreatment dose. Shown are the fever responses (ΔT-3) to lipid A/BSA (day 2, 100 MPD-3/kg, i.v.) of groups of rabbits that had been pretreated (day 0, i.v.) with 1 (A), 10 (B), and 100 (C) MPD-3 of lipid A/BSA per kg. Lipid A antiserum (0.25 ml/kg) was given on day 1.*

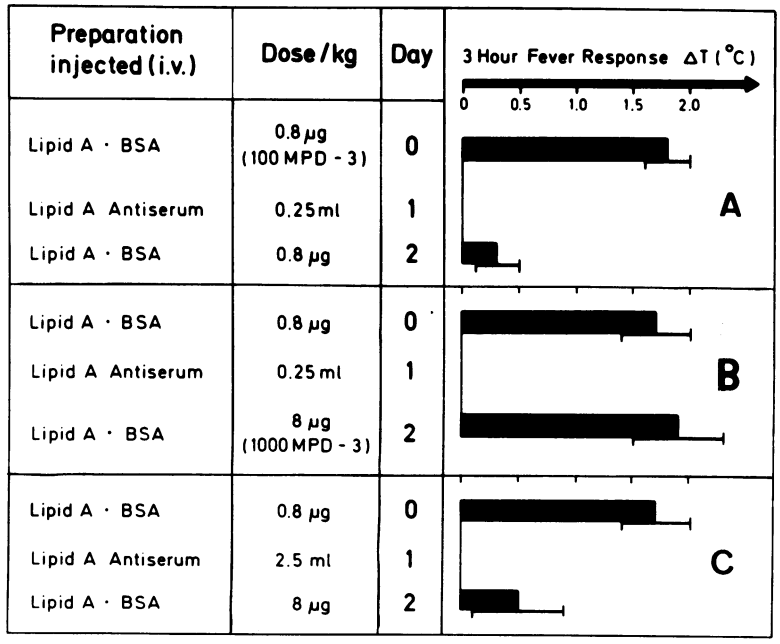

FIG. 8. *Dependency of the degree of lipid A antiserum-mediated fever tolerance on the challenge dose and the amount of antiserum transferred. Shown are the fever responses (ΔT-3) in groups of rabbits pretreated with lipid A/BSA (day 0, 100 MPD-3/kg, i.v.) and lipid A antiserum (day 1, 0.25 ml/kg, i.v.) to 100 (A) and 1,000 (B) MPD-3 of lipid A/BSA per kg (day 2, i.v.). Panel C shows that larger amounts of lipid A antiserum (2.5 ml/kg, day 1, i.v.) suppressed fever induced by 1,000 MPD-3 of lipid A/BSA per kg (day 2, i.v.) in lipid A/BSA-pretreated (100 MPD-3/kg, day 0, i.v.) rabbits.*

A/BSA per kg, no protection was seen (Fig. 8B). With larger amounts (2.5 ml/kg) of antiserum, however, protection could be achieved against challenge with 1,000 MPD-3 of lipid A/BSA per kg (Fig. 8C). Thus, the degree of fever immunity in the system described is dependent on the preparative and the challenge doses as well as on the amount of antiserum transferred.

Cross-protection. The question was then asked whether lipid A antiserum would mediate protection against fever induced by S- and R-form lipopolysaccharides. Rabbits were pretreated with 100 MPD-3 of lipid A/BSA per kg and, after receiving lipid A antiserum, challenged on day 2 with 100 MPD-3/kg doses of lipopolysaccharides from *Salmonella abortus-equi* (S), *S. typhi* (S), *S. minnesota* (Re), *Shigella flexneri* 5b (Re), and *E. coli* (Re). Cross-protection was readily achieved to all lipopolysaccharides tested (Fig. 9). In another set of experiments, it was shown that rabbits pretreated with *S. abortus-equi* lipopolysaccharide (100 MPD-3/kg, day 0) and lipid A antiserum (2.5 ml/kg, day 1) were protected as well against lipid A/BSA (Fig. 10C) as against lipopolysaccharide-induced fever (Fig. 10B). It therefore seems that lipid A antiserum mediates protection against pyrogenicity of both lipopolysaccharides and lipid A in rabbits that have been pretreated by lipopolysaccharide or lipid A.

Since lipid A is known to be a B-lymphocyte mitogen (1), capable of inducing a polyclonal antibody stimulation, the question arose whether lipid A antiserum contained O- and R-specific antibodies, naturally or stimulated by lipid A as a mitogen. These immunoglobulins could be responsible for the protection against lipopolysaccharide fever. To test this possibility, lipid A antiserum was analyzed for the

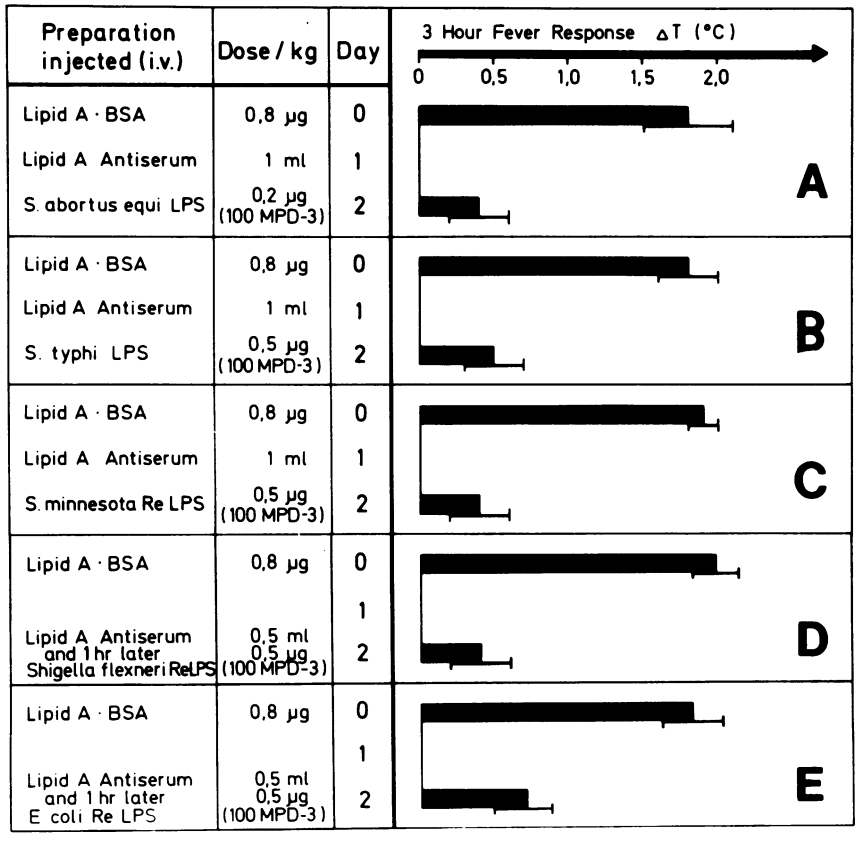

FIG. 9. *Cross-tolerance to lipopolysaccharide fever induced by lipid A antiserum in lipid A/BSA-pretreated rabbits. Shown are the fever responses (ΔT-3) in groups of rabbits pretreated with Salmonella lipid A/BSA (day 0, i.v.) and Salmonella lipid A antiserum to 100 MPD-3/kg doses (day 2) of lipopolysaccharides from S. abortus-equi (A), S. typhi (B), S. minnesota (C), Shigella flexneri (D), and E. coli (E). Doses of lipid A/BSA and lipopolysaccharides used as well as amount and time of transfer of antiserum are as indicated in the figure.*

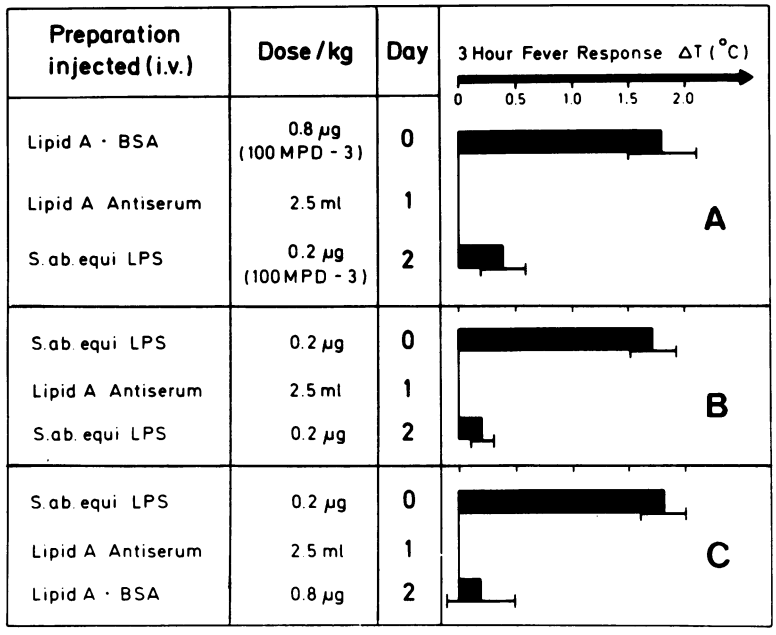

FIG. 10. *Reciprocal cross-tolerance of lipid A/BSA (lipopolysaccharide) fever induced by lipid A antiserum in lipopolysaccharide (lipid A/BSA)-pretreated rabbits. Shown are the fever responses (T-3) of groups of rabbits, where the pretreatment (day 0) and challenge (day 2) injections consisted of lipid A/BSA and S. abortus-equi lipopolysaccharide (A), lipopolysaccharide and lipopolysaccharide (B), and lipopolysaccharide and lipid. A/BSA (C). Lipid A antiserum (2.5 ml/kg) was given on day 1. The doses of lipid A/BSA and lipopolysaccharide used are as indicated in the figure.*

presence of O- and R-specific antibodies (*S. typhi*), and a hemolytic anti-*S. typhi* activity of 1:160 was found. This lipid A antiserum, when given to *S. typhi* pretreated rabbits, conferred, as expected, good protection against *S. typhi* lipopolysaccharide-induced fever (Fig. 11D). To a second group of rabbits, also pretreated with *S. typhi* lipopolysaccharide (day 0), anti-*S. typhi* antiserum (1 ml/kg) was given on day 1. This antiserum, which had been prepared in rabbits against nonpyrogenic, lipid A-free cross-linked polysaccharide from *S. typhi* lipopolysaccharide, exhibited a hemolytic anti-*S. typhi* activity of 1:256. On challenge of the treated rabbits with 100 MPD-3 of *S. typhi* lipopolysaccharide per kg on day 2, a normal fever response was obtained (Fig. 11C). Since lipid A antiserum conferred significantly better protection to pyrogenicity of *S. typhi* lipopolysaccharide than did the homologous antiserum in the doses used, we conclude that polysaccharide-specific antibodies, possibly present in lipid A antiserum, play a minor role, if any, in the mediation of cross-protection. The upper panels of Fig. 11 represent control experiments in which *S. typhi* lipopolysaccharide-treated rabbits (day 0) were given no serum (Fig. 11A) or NRS (Fig. 11B) on day 1. In both cases, a normal fever response was obtained on challenge (day 2) with *S. typhi* lipopolysaccharide.

Specificity of protection. Specificity of this system of fever immunity could be demonstrated in several ways.

First, the protective factor could be removed from the antiserum with lipid A. Lipid A antiserum was absorbed with erythrocytes coated with alkali-treated lipid A. The absorbed serum (hemolytic titer < 1:4), which was not pyrogenic, was transferred (day 1) to lipid A/BSA-pretreated (day 0) rabbits, and the febrile response to 100 MPD-3 of lipid A/BSA per kg was determined on day 2. A fever response not significantly different from that of controls was obtained (Fig. 12B), indicating that the antiserum after absorption had lost the ability to confer immunity. This absorption was lipid A specific, since absorption of O antiserum (*S. abortus-equi*) with lipid A did not result in any loss of anti-O serological activity (F. Jay and C. Galanos, unpublished data). Absorption of the antiserum with nonsensitized erythrocytes did not alter its protective power (Fig. 12C). The control experiment with untreated antiserum is shown in the upper panel of Fig. 12.

The specificity of *Salmonella* lipid A antiserum became obvious when it was tested with *Chromobacterum violaceum* lipopolysaccharide. This pyrogen was investigated because

Preparation injected (i.v.)	Anti-S.typhi Activity (Rec.Titers)	Dose / kg	Day
S typhi LPS		0,5 µg (100 MPD-3)	0
—	—		1
S typhi LPS		0,5 µg	2
S typhi LPS		0,5 µg	0
NRS	20	1 ml	1
S typhi LPS		0,5 µg	2
S typhi LPS		0,5 µg	0
O-Antiserum	256	1 ml	1
S typhi LPS		0,5 µg	2
S typhi LPS		0,5 µg	0
Lipid A Antiserum	160	1 ml	1
S typhi LPS		0,5 µg	2

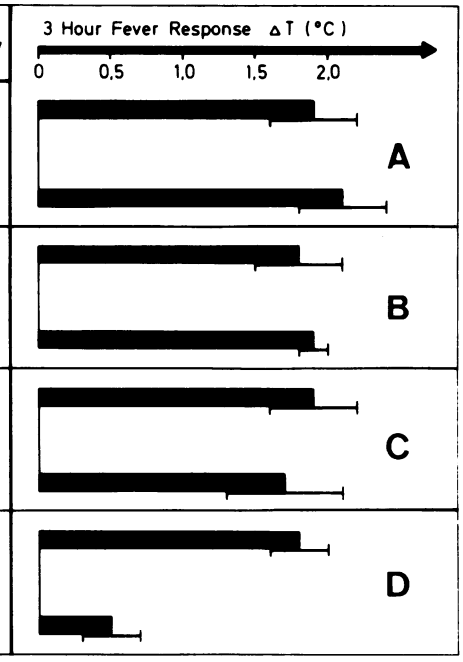

FIG. 11. *Role of O antibodies in tolerance to lipopolysaccharide fever induced by lipid A antiserum in lipid A/BSA-pretreated rabbits. Shown are the fever responses (ΔT-3) of groups of rabbits to S. typhi lipopolysaccharide (day 2, 100 MPD-3/kg, i.v.) pretreated on day 0 with lipopolysaccharide (100 MPD-3/kg, i.v.) and on day 1 with no serum (A), NRS (B), O antiserum (C), and lipid A antiserum (D). Also shown is the anti-S. typhi activity (reciprocal hemolytic titers) of the sera tested. Doses of lipopolysaccharide used and amounts of sera transferred are as indicated in the figure.*

Preparation injected (i.v.)	Dose / kg	Day
Lipid A · BSA	0.8 µg (100 MPD - 3)	0
Lipid A Antiserum (Titer 1 : 2024)	0.25 ml	1
Lipid A · BSA	0.8 µg	2
Lipid A · BSA	0.8 µg	0
Lipid A Antiserum absorbed with Lipid A - OH sensitized SRBC'S (Titer <1 : 4)	0.25 ml	1
Lipid A · BSA	0.8 µg	2
Lipid A · BSA	0.8 µg	0
Lipid A Antiserum absorbed with SRBC'S (Titer 1 : 2024)	0.25 ml	1
Lipid A · BSA	0.8 µg	2

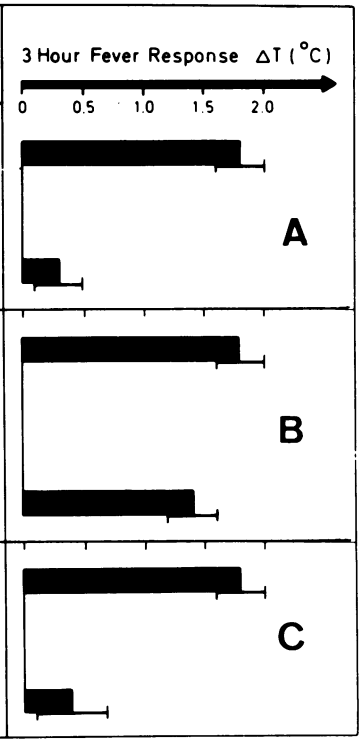

FIG. 12. *Effect of lipid A antiserum absorbed with alkali-treated lipid A on lipid A/BSA pyrogenicity in lipid A/BSA-pretreated rabbits. Shown are the fever responses of groups of rabbits (ΔT-3) to lipid A/BSA (day 2, 100 MPD-3/kg). Rabbits were pretreated with lipid A/BSA (day 0, 100 MPD-3/kg, i.v.) and on day 1 with 0.25 ml of nonabsorbed serum per kg (A), with serum absorbed with erythrocytes coated with alkali-treated lipid A (B), and with serum absorbed with erythrocytes (C).*

Watson and Kim (25) had shown that rabbits made tolerant to *E. coli* O8 lipopolysaccharide did not exhibit cross-tolerance against *C. violaceum* lipopolysaccharide. They further found that animals made tolerant to *C. violaceum* were tolerant to *E. coli* (nonreciprocal cross-reaction). These findings were confirmed in the present study with lipid A antiserum. In lipid A/BSA-pretreated rabbits (day 0), lipid A antiserum did not confer protection to pyrogenicity of *C. violaceum* lipopolysaccharide (Fig. 13C). However, when animals were pretreated with 100 MPD-3 of *C. violaceum* lipopolysaccharide per kg, transfer of lipid A antiserum provided substantial protection against lipid A/BSA fever (Fig. 13A).

Finally, attempts were made to abolish fever caused by nonendotoxic pyrogens such as dsRNA by using lipid A antiserum. Rabbits were prepared in the usual way with lipid A/BSA (100 MPD-3/kg; day 0) and lipid A antiserum (0.25 ml/kg; day 1). The animals were then injected (day 2) with pyrogenic dsRNA in doses of 50 and 5 μg/kg, respectively. The fever responses obtained (Fig. 14A and B) were not significantly different from those observed after injection of the same doses of dsRNA into nontreated rabbits (Fig. 14, upper panel). The results of reciprocal experiments are shown in Fig. 15. Groups of rabbits received pyrogenic dsRNA in the doses indicated (10-fold dilutions from 50 to 0.005 μg/kg) on day 0. After antiserum administration (day 1), the animals were challenged (day 2) with 100 MPD-3/kg doses of lipid A/BSA. All animals showed normal fever responses, indicating that preinjection of dsRNA did not allow the lipid A antiserum to express its protective power against lipid A fever. The fever immunity mediated by lipid A antiserum therefore was lipid A (endotoxin) specific with regard to both preparation (day 0) and challenge (day 2).

Prevention of the local Shwartzman reaction. The biological significance of lipid A antiserum was also evaluated in a second test system, namely lipopolysaccharide-induced skin necrosis (local Shwartzman reaction). Groups of New Zealand white rabbits were prepared for the reaction by intracutaneous administration of *S. abortus-equi* lipopolysaccharide at four different skin sites (40 μg/site). Twenty hours later, the prepared animals were given NRS (4

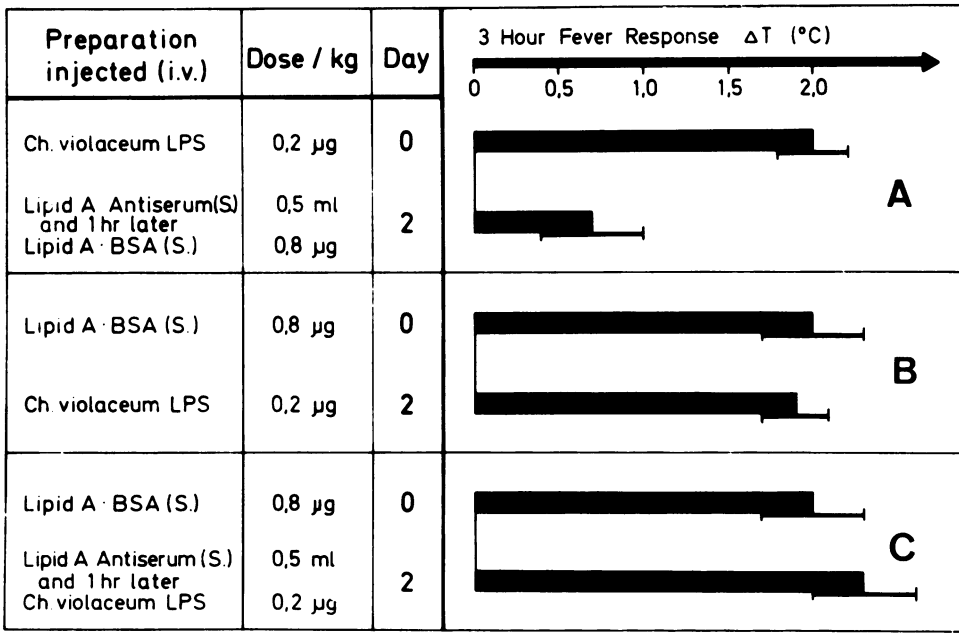

FIG. 13. *Inability of lipid A antiserum (Salmonella) to suppress fever induced by Chromobacterium violaceum lipopolysaccharide in lipid A/BSA-pretreated rabbits. Shown are the fever responses (ΔT-3) to C. violaceum lipopolysaccharide (day 2, 100 MPD-3/kg, i.v.) in groups of rabbits pretreated on day 0 with Salmonella lipid A/BSA (100 MPD-3/kg, i.v.) and Salmonella lipid A antiserum (C) or no serum (B). (A): After pretreatment (day 0) with C. violaceum lipopolysaccharide and lipid A antiserum (Salmonella), rabbits were challenged with lipid A/BSA (Salmonella). Doses of lipid A/BSA and lipopolysaccharide as well as amount and time of transfer of antiserum are as indicated in the figure.*

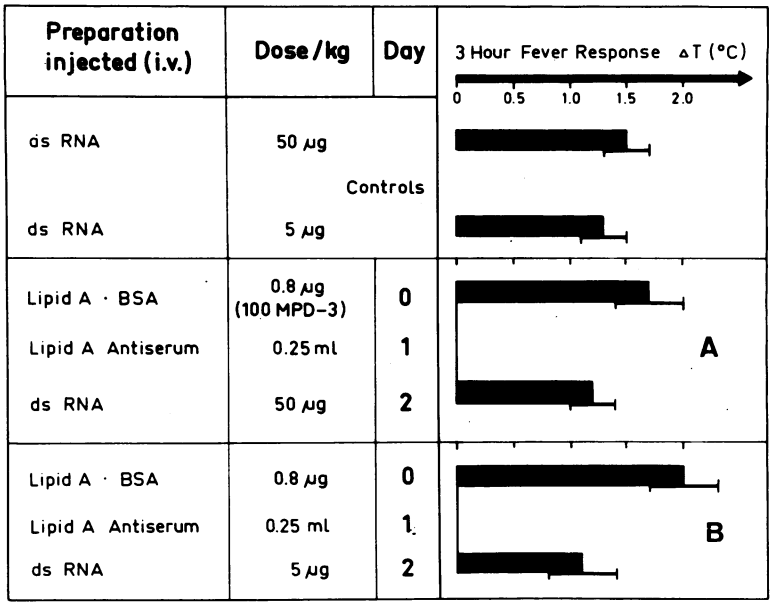

FIG. 14. *Inability of lipid A antiserum to mediate tolerance to fever induced by pyrogenic dsRNA in lipid A/BSA-pretreated rabbits. Shown are the fever responses (ΔT-3) in groups of rabbits pretreated with lipid A/BSA (day 0, 100 MPD-3/kg, i.v.) and lipid A antiserum (day 1, 0.25 ml/kg, i.v.) to a challenge (i.v.) of dsRNA on day 2 (50 μg/kg [A]; 5 μg/kg [B]). The upper panel shows the fever responses of nontreated rabbits to pyrogenic dsRNA (50 and 5 μg/kg).*

ml, i.v.) or lipid A antiserum (4 ml, i.v.). The reaction was provoked 2 h after serum transfer by i.v. injection of either lipid A/BSA (20 μg) or lipopolysaccharide (*S. abortus-equi*, 10 μg; *S. typhi*, 20 μg).

Lipid A/BSA did not elicit skin necrosis in animals pretreated with lipid A antiserum (Table 2). When *S. abortus-equi* or *S. typhi* lipopolysaccharides were used for challenge in antiserum-treated rabbits, 9 of 10 and 9 of 12 animals were protected, respectively, whereas about 90% of control rabbits, which had received NRS, reacted positively. Thus, lipid A antiserum exhibits a significant protective effect on lipid A/BSA- and lipopolysaccharide-induced skin necrosis.

DISCUSSION

The experiments described in the present paper were performed to evaluate possible antiendotoxic properties of lipid A antiserum. In a first series of experiments, using lipid A-induced fever as a test system, it was found that the antiserum exhibits no significant direct neutralizing activity. It was then shown that lipid A antiserum confers protection to lipid A fever in rabbits that had been pretreated with a single dose of lipid A or lipopolysaccharide. This unexpected finding indicated that the antiserum possesses the potential to mediate resistance to lipid A fever, but that this activity is only expressed in animals previously exposed to lipid A. In this respect, lipid A antiserum differs from antisera specific to the polysaccharide portion (O-specific chain and core) of lipopolysaccharides. With these sera, suppression of lipopolysaccharide pyrogenicity is achieved by incubation (19). Furthermore, protection to lipopolysaccharide-induced fever is, after passive transfer, observed in normal rabbits without pretreatment (10). This indicates that the mechanisms underlying the antiendotoxic actions of anti-lipid A or anti-polysaccharide antisera are distinct. Why pretreatment is necessary in lipid A antiserum-mediated protection is not understood. It is evident, however, that the pronounced effect of the antiserum on lipid A activity is not merely due to a classical antitoxin-toxin interaction. Apparently there are, besides humoral factors (present in lipid A antiserum), other factors, perhaps cellular, involved in the protection system described here.

With *Salmonella* lipid A antiserum, protection was seen not only against pyrogenicity of *Salmonella* lipid A but also against that of S- and R-form lipopolysaccharides from *Salmonella* species. Furthermore, cross-protection to lipopolysaccharides from other bacterial groups (*Shigella flexneri*, *E. coli*) was demonstrated. This cross-protection was to be expected in view

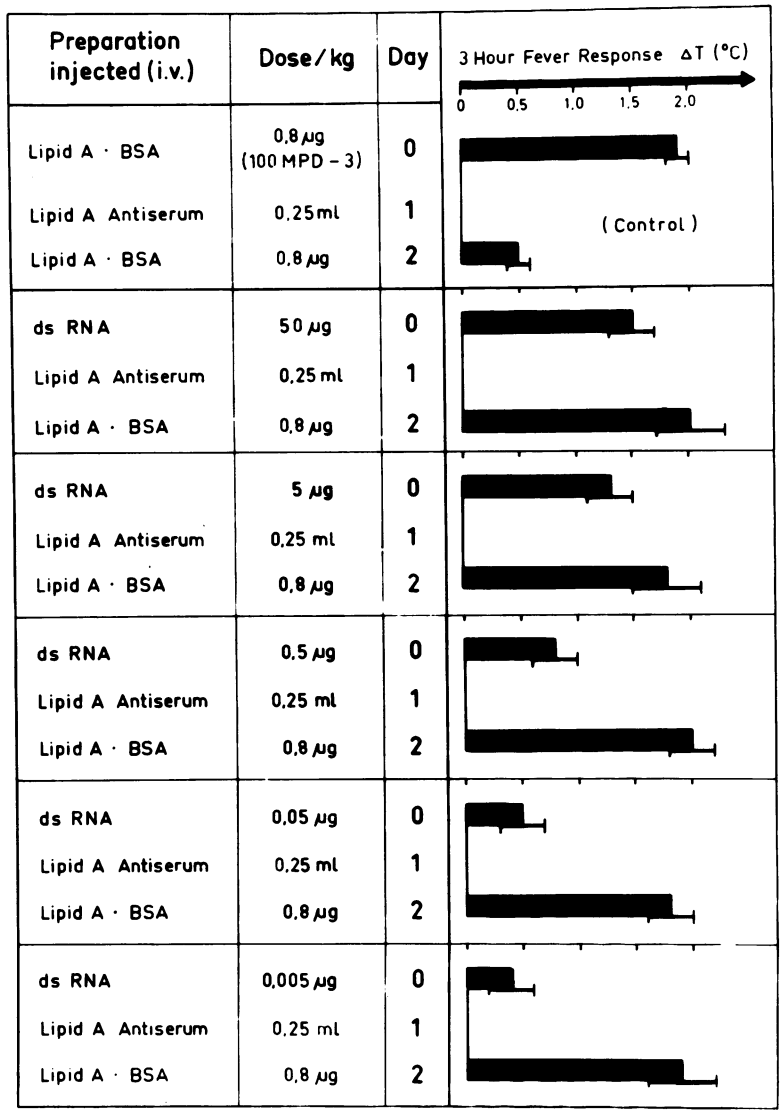

FIG. 15. *Inability of lipid A antiserum to mediate tolerance to lipid A/BSA fever in rabbits pretreated with pyrogenic dsRNA. Shown are the fever responses (ΔT-3) to lipid A/BSA (day 2, 100 MPD-3/kg, i.v.) in groups of rabbits pretreated (day 0) with graded doses (10-fold dilutions from 50 to 0.005 µg/kg, i.v.) of dsRNA and lipid A antiserum (day 1, 0.25 ml/kg, i.v.). The upper panel shows a control experiment with lipid A/BSA pretreatment (compare Fig. 4A, 7C, 8A, and 12A).*

of the structural similarities and serological cross-reactions existing between lipid A's of various bacterial groups (11). However, fever elicited by pyrogenic dsRNA could not be inhibited. Also, rabbits pretreated with dsRNA were, after transfer of lipid A antiserum, not resistant to lipid A fever. These results point to a lipid A specificity of the protection system described. Since lipid A is common to most pyrogenic lipopolysaccharides (endotoxins), this specificity could also be termed "interendotoxin" specificity (9). Lipid A specificity possibly refers to two distinct features of lipid A. The capacity of lipid A to act as a preparative agent (day 0) could be related to its "endotoxic" properties. The specificity seen with the challenge (day 2 after serum transfer), however, is related to the antigenic properties of lipid A. Thus, the structures in lipid A determining endotoxicity may be different from those determining (serological) specificity. Because of its lipid A (endotoxin) specificity, the

present system could be used to discriminate between fever caused by endotoxin (lipopolysaccharide, lipid A) and that induced by other (non-endotoxin) pyrogens.

The necessity of performing reciprocal cross-testing, however, is indicated by the experiments involving lipopolysaccharide from *C. violaceum*. Rabbits pretreated with this lipopolysaccharide could be protected with lipid A antiserum (*Salmonella*) against subsequent *Salmonella* lipid A challenge. But *Salmonella* lipid A-pretreated animals, despite passive transfer of lipid A antiserum (*Salmonella*), reacted to challenge with *C. violaceum* lipopolysaccharide with a normal fever response. This nonreciprocal cross-protection had already been noted by Watson and Kim, who studied actively induced endotoxin tolerance (25). It is tempting to assume that the lack of cross-protection is due to a structural difference of lipid A of *C. violaceum* from that of enterobacterial lipid A. Results of first chemical analyses seem to support this hypothesis (S. Hase and E. Th. Rietschel, unpublished data).

The duration of protection in the system described was not studied systematically. Duration depends, however, on the preparative lipid A injection, the effect of which gradually vanishes within 5 days. Therefore, lipid A-pretreated rabbits tested on day 6 react like nontreated (normal) animals. Evidently, the duration of protection also depends on the presence of a protective factor. Studies on the elimination of hemolytic lipid A antibodies in rabbits after application of lipid A antiserum (10 ml, i.v.; titer, 1:10,000) showed that after 8 days the titer was still significant (hemolytic titers, 1:256), whereas after 16 days no activity could be detected. Similar kinetics of elimination were found in lipid A-pretreated rabbits.

Experiments concerning the time of transfer of lipid A antiserum showed that it can be given simultaneously with the preparative (day 0) and the challenge (day 2) doses of lipid A/BSA or at times between the injections. However, when lipid A antiserum was given 1 h after the lipid A/BSA challenge, no protection was obtained, indicating that the antiserum has no effect on the lipid A-induced mechanisms leading to fever, but rather that it inhibits lipid A from triggering these mechanisms.

The nature of the protecitve factor in lipid A antiserum remains to be elucidated. As shown above, it can be removed from the antiserum by erythrocytes coated with alkali-treated lipid A and thus appears to be lipid A specific. It should be mentioned that on absorption of lipid A antiserum with lipid A (coated on erythrocytes), the detectable serological activity (anti-lipid A antibody) was completely removed. Despite this, the absorbed serum still conferred a certain degree of protection as compared with the effect of NRS (Fig. 12B and 4B; $P < 0.025$). Nevertheless, it is evident from Fig. 12A and 12B that the absorbed serum was significantly less protective than nonabsorbed lipid A antiserum ($P < 0.005$).

As preliminary experiments show, the protective factor can be precipitated with half-saturated ammonium sulfate, suggesting that it might be identical with immunoglobulin. However, in sera of lipid A-pretreated rabbits that had received small amounts of lipid A antiserum (0.25 to 0.5 ml/kg) and were resistant to lipid A fever, no hemolytic lipid A antibodies could be demonstrated. This could mean either that very small amounts of antibodies, not detectable by the method available, provide protection or that the protective immunoglobulin is distinct from hemolytic lipid A antibody (mainly immunoglobulin M). Since Greisman et al. (10) have demonstrated the existence of cytophilic anti-lipopolysaccharide antibodies, the possibility should also be considered that the protective factor acts (in lipid A-pretreated rabbits) primarily on a cellular level, and that it is therefore not detectable in sera of pretreated and passively immunized rabbits.

Kim and Watson have postulated that antibodies specific to the common toxic moiety of

TABLE 2. *Prevention of the local Shwartzman reaction in rabbits with lipid A antiserum*

Prepn (intracutaneous)		Challenge (intravenous)		Incidence of positive Shwartzman reactions after:[a]	
LPS	Dose (μg)	LPS (lipid A)	Dose (μg)	NRS	Lipid A antiserum
Salmonella abortus-equi	40	Lipid A/BSA	20[b]	6/6[c]	0/6
S. abortus-equi	40	*S. abortus-equi*	10	5/6	1/10
S. abortus-equi	40	*S. typhi*	20	9/10	3/12

[a] NRS (4 ml) or lipid A antiserum (4 ml) was given 2 h before the challenge injection of lipid A or lipopolysaccharide (LPS).

[b] Based on the lipid A content of the complex.

[c] Number of rabbits with positive reaction/number of rabbits tested.

lipopolysaccharides could mediate tolerance and especially cross-tolerance, which was induced by several daily injections of lipopolysaccharide or lipid A (13–15, 25). These antibodies were thought to be induced by the daily injections of lipopolysaccharides or lipid A. In sera of rabbits rendered actively tolerant with lipid A/BSA (22), we have detected small but significant hemolytic anti-lipid A activity (titers, 1:4 to 1:64). The latter finding seems to support their hypothesis. In the light of the experiments described in this paper, in actively induced tolerance the daily administration of lipopolysaccharide or lipid A could mean optimal "preparation" of the test animals. Lipid A antibodies are engendered concomitantly with the repeated injections, which would mediate tolerance and cross-tolerance in the "pretreated" animals. It is worthwhile to note that tolerance induced with several daily lipopolysaccharide injections exhibits lipid A specificity similar to that described in the present paper (14, 25). On the other hand, Braude et al. (4), Milner and Rudbach (Bacteriol. Proc., p. 96, 1968), Milner (18), and Greisman et al. (8, 9) have demonstrated that a single injection of lipopolysaccharide (day 0) will render rabbits transitorily tolerant to a subsequent (day 1) lipopolysaccharide challenge. This "early refractory state" also shows lipid A specificity (8, 9, 12, 18, 24; Milner and Rudbach, Bacteriol. Proc., p. 96, 1968). Assuming that normal rabbits have small amounts of lipid A-specific factors not detectable by the methods available, it can be expected that rabbits, after a "preparative" endotoxin injection (day 0), will be protected against a second endotoxin challenge (administered 24 h later).

It is tempting to speculate that all three types of fever tolerance, early refractory state, cross-tolerance actively induced by several endotoxin injections, and the protection system described in the present communication, are mediated by the same mechanisms. In these mechanisms, as discussed, humoral factors (specific to lipid A) play an essential role. However, other factors, induced or activated by a single or several endotoxin injections, are also involved; their nature remains to be analyzed.

Evidence was provided in this study that the local Shwartzman reaction, provoked with lipid A or lipopolysaccharides, could also be prevented by lipid A antiserum. Thus, two typical endotoxin effects, fever and local skin necrosis, can be suppressed by the antiserum, a finding which indicates that lipid A antiserum may also provide protection against other endotoxin activities. Its possible ability to prevent endotoxin-induced leucopenia and irreversible shock is presently being investigated.

ACKNOWLEDGMENTS

The skillful technical assistance of Ulrike Pflugfelder and Christine Strohmeier is gratefully acknowledged. Thanks are due to Karla Klettner and Ellen Ruschmann for serological assays of anti-lipid A activity in sera. Appreciation is expressed to O. Lüderitz and O. Westphal for their interest and encouragement. We also thank G. Greer, S. Greisman, and D. W. Watson for advice and criticism.

This investigation was supported by grants from the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Andersson, I., F. Melchers, C. Galanos, and O. Lüderitz.** 1973. The mitogenic effect of lipopolysaccharide on bone marrow-derived mouse lymphocytes. Lipid A as the mitogenic part of the molecule. J. Exp. Med. 137:943–953.
2. **Braude, A. J., and H. Douglas.** 1972. Passive immunization against the local Shwartzman reaction. J. Immunol. 108:505–512.
3. **Braude, A. J., H. Douglas, and C. Davies.** 1973. Treatment and prevention of intravascular coagulation with antiserum to endotoxin. J. Infect. Dis. 128(Suppl.):S157–164.
4. **Braude, A. J., M. Zalesky, and H. Douglas.** 1958. The mechanism of tolerance to fever. I. Clin. Invest. 37:880–881.
5. **Galanos, C., O. Lüderitz, and O. Westphal.** 1969. A new method for the extraction of R-lipopolysaccharides. Eur. J. Biochem. 9: 245–249.
6. **Galanos, C., O. Lüderitz, and O. Westphal.** 1971. Preparation and properties of antisera against the lipid A component of bacterial lipopolysaccharides. Eur. J. Biochem. 24:116–122.
7. **Galanos, C., E. Th. Rietschel, O. Lüderitz, and O. Westphal.** 1971. Interaction of lipopolysaccharides and lipid A with complement. Eur. J. Biochem. 19:143–152.
8. **Greisman, S. E., and W. E. Woodward.** 1965. Mechanisms of endotoxin tolerance. III. The refractory state during continuous intravenous infusions of endotoxin. J. Exp. Med. 121:911–933.
9. **Greisman, S. E., E. J. Young, and J. A. Carozza, Jr.** 1969. Mechanisms of endotoxin tolerance. V. Specificity of the early and late phases of pyrogenic tolerance. J. Immunol. 103:1223–1236.
10. **Greisman, S. E., E. J. Young, and B. DuBuy.** 1973. Mechanisms of endotoxin tolerance. VIII. Specificity of serum transfer. J. Immunol. 111:1349–1360.
11. **Hase, S., and E. Th. Rietschel.** 1976. Isolation and analysis of the lipid A backbone. Lipid A structure of lipopolysaccharides from various bacterial groups. Eur. J. Biochem. 63:101–107.
12. **Kanoh, S., H. Kawasaki, and A. Nishio.** 1969. Studies on myxovirus pyrogen. (II). Some factors affecting fever tolerance in rabbits. Biken J. 12:169–180.
13. **Kim, Y. B., and D. W. Watson.** 1965. Modification of host response to bacterial endotoxin. II. Passive transfer of immunity to bacterial endotoxin with fractions containing 19S antibodies. J. Exp. Med. 121:751–759.
14. **Kim, Y. B., and D. W. Watson.** 1966. Role of antibodies in reaction to gram-negative bacterial endotoxins. Ann. N.Y. Acad. Sci. 133:727–745.
15. **Kim, Y. B., and D. W. Watson.** 1967. Biologically active endotoxins from *Salmonella* mutants deficient in O- and R-polysaccharides and heptose. J. Bacteriol. 94:

1320–1326.
16. **Lüderitz, O., Ch. Galanos, V. Lehmann, M. Nurminen, E. Th. Rietschel, G. Rosenfelder, M. Simon, and O. Westphal.** 1973. Lipid A: chemical structure and biological activity. J. Infect. Dis. 128(Suppl.):S9–S21.
17. **Lüderitz, O., O. Westphal, A. M. Staub, and H. Nikaido.** 1972. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides, p. 145–233. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV. Academic Press Inc., New York.
18. **Milner, K. C.** 1973. Patterns of tolerance to endotoxin. J. Infect. Dis. 128(Suppl.):S237–S245.
19. **Radvany, R., N. L. Neale, and A. Nowotny.** 1966. Relation of structure to function in bacterial O-antigens. VI. Neutralization of endotoxic O-antigens by homologous O-antibody. Ann. N.Y. Acad. Sci. 133:763–786.
20. **Rietschel, E. Th.** 1975. Chemical structure and biological activity of endotoxins (lipopolysaccharides) and lipid A. Naunyn-Schmiedeberg's Arch. Pharmacol. 287:73–84.
21. **Rietschel, E. T., C. Galanos, and O. Lüderitz.** 1975. Structure, endotoxicity, and immunogenicity of the lipid A component of bacterial lipopolysaccharides, p. 307–314. *In* D. Schlessinger (ed.), Microbiology–1975. American Society for Microbiology, Washington D.C.
22. **Rietschel, E. T., Y. B. Kim, D. W. Watson, C. Galanos, O. Lüderitz, and O. Westphal.** 1973. Pyrogenicity and immunogenicity of lipid A complexed with bovine serum albumin or human serum albumin. Infect. Immun. 8:173–177.
23. **Rietschel, E. Th., and O. Lüderitz.** 1975. Chemical structure of lipopolysaccharides and endotoxin immunity. Z. Immunitaetsforsch. 149:201–213.
24. **Urbaschek, B., and A. Nowotny.** 1968. Endotoxin tolerance induced by detoxified endotoxin (endotoxoid). Proc. Soc. Exp. Biol. Med. 127:650–652.
25. **Watson, D. W., and Y. B. Kim.** 1963. Modifications of host response to bacterial endotoxins. I. Specificity of pyrogenic tolerance and role of hypersensitivity in pyrogenicity, lethality and skin reactivity. J. Exp. Med. 118:425–446.
26. **Westphal, O., O. Lüderitz, and F. Bister.** 1952. Über die Extraktion von Bakterien mit Phenol-Wasser. Z. Naturforsch. 7b:148–155.