

A 28,000-Dalton Protein of Normal Mouse Serum Binds Specifically to the Inner Core Region of Bacterial Lipopolysaccharide

LORE BRADE AND HELMUT BRADE*

Forschungsinstitut Borstel, D-2061 Borstel, Federal Republic of Germany

Received 2 May 1985/Accepted 21 August 1985

Normal mouse serum was found to contain a protein, referred to here as factor, which binds to the inner core region of lipopolysaccharides (LPSs) of various bacterial families. Since factor-LPS interactions resulted in activation of guinea pig complement, factor activity could be assayed by a passive hemolysis test with sheep erythrocytes coated with LPS or lipid A from *Acinetobacter calcoaceticus* (which was found earlier to bind particularly well to factor). Factor was purified by G-50 and hydroxyapatite chromatography whereby the specific hemolytic activity was enriched 1,675-fold. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions revealed the presence of a 28,000-dalton protein as the main band. The identity of this band was determined by absorption experiments with LPS-coated sheep erythrocytes or latex beads, whereby the 28,000-dalton band disappeared after specific absorption and could be recovered from the absorbent. The binding specificity of factor was determined in a passive hemolysis inhibition assay with defined oligosaccharides representative for the inner core region of LPS. Thus, the di- and trisaccharides α -D-mannoheptopyranosyl-(1 \rightarrow 5)-2-keto-3-deoxy-D-mannooctonic acid and α -D-mannoheptopyranosyl-(1 \rightarrow 3)- α -D-mannoheptopyranosyl-(1 \rightarrow 5)-2-keto-3-deoxy-D-mannooctonic acid, respectively, were able to inhibit binding of factor to LPS. The results are in accordance with our earlier observation that the heptose-2-keto-3-deoxy-D-mannooctonic acid region represents a common antigen of bacterial LPS. Rabbit hyperimmune serum directed against this common antigen and purified factor was found to exhibit the same specificity for LPS. Factor activity was followed in mice in vivo after injection of LPS; it disappeared completely 15 min after the injection of LPS and reappeared within 1 h.

In previous communications, we have reported on a new type of common antigen, referred to as common lipopolysaccharide (LPS) specificity, which is located in the inner core region of bacterial LPS (4, 5). This antigen is particularly well exposed in the LPS and lipid A of *Acinetobacter calcoaceticus* (3, 4), a member of the *Neisseriaceae* family; however, immunochemical investigations have shown that it is present in all S- and R-form LPSs of different bacterial families except in those enterobacterial strains having the Re chemotype (5). It was further demonstrated that, for the expression of this antigenic determinant, 2-keto-3-deoxy-D-mannooctonic acid (KDO) and one neutral sugar attached to it were required (2, 5).

In addition, this determinant is immunogenic for rabbits, since immunization with killed *A. calcoaceticus* bacteria led to the production of antibodies which were highly specific for the common LPS specificity (4). Moreover, we have found earlier that normal serum of many mammalian species including humans, was able to lyse sheep erythrocytes (SRBC) sensitized with LPS or lipid A from *A. calcoaceticus* by recognizing the common LPS specificity (4). The lysis was shown to be dependent on the activation of complement by the classical pathway (unpublished result). This hemolytic activity was particularly high in mice and rats, where titers of up to 4,000 were found. The present investigation was carried out to elucidate the nature of this naturally occurring, LPS-binding serum activity. It will be shown that, in the mouse, this hemolytic activity is not immunoglobulin, but a low-molecular-weight protein, which after interaction with a specific ligand (KDO region of LPS) activates complement as measured by a hemolytic test system. This protein is referred to here as factor.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA), essentially fatty acid free, was obtained from Sigma Chemical Co. (Munich, Federal Republic of Germany), as were the oligosaccharides maltose, raffinose, stachyose, turanose, palatinose, gentiobiose, and *N,N*-diacetyl-chitobiose. 2-Mercaptoethanol was purchased from Serva (Heidelberg, Federal Republic of Germany). Trypsin was from Boehringer (Mannheim, Federal Republic of Germany), and hydroxyapatite was from Calbiochem (La Jolla, Calif.). The ammonium salt of KDO was kindly provided by F. M. Unger, Sandoz-Forschungsinstitut (Vienna, Austria).

Bacterial LPS and lipid A. LPS and lipid A were prepared from *A. calcoaceticus* as described previously (3). Lipid A was obtained from LPS of an *Escherichia coli* Re mutant, strain F515, after hydrolysis in 0.1 M acetate buffer (100°C for 1 h). Alkali treatment was performed in 0.25 M NaOH (56°C for 1 h) by the method of Neter (27). α -D-Mannoheptopyranosyl-(1 \rightarrow 5)-KDO and α -D-mannoheptopyranosyl-(1 \rightarrow 3)- α -D-mannoheptopyranosyl-(1 \rightarrow 5)-KDO were prepared from the LPS of *Salmonella minnesota* strains R4 (Rd₂P⁻ mutant) and R7 (Rd₁P⁻ mutant), respectively, after hydrolysis (acetate buffer, pH 4.4, 0.1 M; 100°C for 1 h) and purification on Bio-Gel P-2 (110 by 2.5 cm; Bio-Rad Laboratories, Munich, Federal Republic of Germany) with water as the eluant. The linkages were determined by gas-liquid chromatography-mass spectrometry of the reduced and permethylated derivatives (H. Brade, H. Moll, and E. T. Rietschel, Biomed. Mass Spectrom., in press). The anomeric configurations were investigated by nuclear magnetic resonance spectroscopy (F. M. Unger and H. Brade, unpublished results).

Animal sera. Normal mouse serum (NMS) was obtained from 10 to 14-week-old female NMRI mice by exsanguina-

* Corresponding author.

tion from the axillary vein under ether anesthesia (kindly provided by K.-D. Hungerer, Behringwerke Marburg, Federal Republic of Germany). The blood was allowed to clot for 30 min at 37°C, followed by centrifugation in the cold. The serum was absorbed with SRBC and stored at -20°C. Serum was also obtained from different inbred strains of mice from the animal stock of the Max-Planck-Institut für Immunbiologie (Freiburg, Federal Republic of Germany). Rabbit hyperimmune serum against the common LPS specificity was prepared as described earlier (4). From this serum, immunoglobulin M (IgM) antibodies were prepared by gel filtration on Sephadex G-200 (1.5 by 100 cm).

Liquid chromatography. Gel permeation chromatography was performed on Sephadex G-50 fine and G-200 and Sephacryl 200 and 300 (Pharmacia Freiburg, Federal Republic of Germany), Bio-Gel P-10 (Bio-Rad), and controlled porous glass (5.9 nm; Serva). The effluent buffer was Tris hydrochloride (100 mM, pH 8.0) in 250 mM NaCl. All effluents were monitored by the passive hemolysis test for hemolytic activity, and the UV absorbance was recorded at 280 nm.

Protein determination and SDS-PAGE. Protein was determined by the method of Lowry et al. (24). Samples containing low amounts of protein were precipitated with trichloroacetic acid (TCA; 10%, wt/vol). Dilute samples were concentrated in the same way for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed by the method of Laemmli (23) with a 14% gel stained with Coomassie brilliant blue G-250 after electrophoresis. Bovine serum albumin (66 kilodaltons [kDa]), ovalbumin (45 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and lactalbumin (14 kDa) were obtained from Sigma and used as molecular mass standards.

Serological methods. The passive hemolysis test was used to measure the hemolytic activity of NMS, purified factor, and rabbit immune serum (4, 15, 27). SRBC were washed three times in phosphate-buffered saline and sensitized with alkali-treated *A. calcoaceticus* lipid A (lipid A-OH). The optimal dose of antigen for sensitization was determined for each batch of antigen (usually in the range of 20 to 80 µg per 0.2 ml of packed SRBC). Sensitization was achieved by incubating packed SRBC (0.2 ml) in phosphate-buffered saline (4 ml) with 40 µg of *A. calcoaceticus* lipid A-OH (in 20 µl of water) at 37°C for 30 min. The antigen-coated cells were washed three times in phosphate-buffered saline and finally suspended in 40 ml of Veronal-buffered saline (VBS) to give a 0.5% suspension. The test was either performed in microtiter trays (semiquantitative assay) or in test tubes (quantitative assay). In the semiquantitative assay, serial twofold dilutions of serum in VBS (50 µl) were incubated with antigen-coated SRBC (50 µl of a 0.5% suspension in VBS) and guinea pig complement (25 µl of a 1:20 dilution in VBS, absorbed with *A. calcoaceticus* lipid A-OH) at 37°C for 1 h. After centrifugation, 50% endpoint titers were determined by the naked eye. One hemolytic unit is defined as the amount of serum causing 50% of hemolysis under these test conditions. In the quantitative assay, 100 µl of a serial twofold serum dilution in VBS was incubated with 100 µl of VBS, 25 µl of guinea pig complement (absorbed with *A. calcoaceticus* lipid A-OH and diluted 1:4 in VBS) and 1.0 ml of a 0.5% suspension of antigen-coated SRBC at 37°C for 1 h. After centrifugation, the supernatants were read spectrophotometrically at 546 nm.

Quantitative inhibition experiments were carried out as follows. Serial twofold dilutions of inhibitor in 100 µl of water were incubated (37°C for 15 min) with 100 µl of an appropriate NMS dilution. This serum dilution (usually in

the range of 1:300 to 1:400) was adjusted to cause 80% of hemolysis without the addition of inhibitor. Antigen-coated SRBC (1.0 ml) and complement (25 µl of a 1:4 dilution) were added, and incubation was continued at 37°C for 1 h. After centrifugation, the supernatants were measured at 546 nm in a spectrophotometer. Inhibition is expressed as the percentage of hemolysis compared with a control without inhibitor.

Absorption experiments. Absorption was carried out at 4°C with native SRBC or at 37°C with glutaraldehyde-fixed SRBC to which the respective ligand had been coated (100 µg per 0.2 ml of packed SRBC). Absorption was also done with antigen-coated latex particles. To 100 µl of a latex suspension (Bacto-Latex 0.81; Difco Laboratories, Detroit, Mich.), 50 µg of antigen in saline (1,400 µl) was added and incubated at room temperature for 1 h, followed by two cycles of washing and centrifugation (10 mM Tris hydrochloride [pH 8.0]; 10,000 × g for 30 min). Active sites were blocked with 0.1% BSA in the same buffer (room temperature for 1 h). After two additional steps of washing, the particles were sedimented by centrifugation and used for absorption of factor activity (see Results).

RESULTS

Purification of factor from NMS. NMS with a hemolytic activity of 80×10^3 hemolytic units per ml was fractionated by gel permeation chromatography on Sephadex G-50. The hemolytic activity eluted within the total volume of the column, indicating either a binding to the gel support or the presence of a low-molecular-weight factor. Determination of protein indicated that 99.8% of the total protein was removed by this first step, and 30% of the hemolytic activity was recovered, resulting in a 400-fold enrichment. The elution pattern is shown in Fig. 1. Fractions containing the hemolytic activity were combined and further purified by hydroxyapatite chromatography. Adsorption was performed batchwise by adding solid hydroxyapatite conditioned with starting buffer until the supernatant was free of hemolytic activity. The hydroxyapatite was transferred to a column and eluted with a linear gradient from 50 to 500 mM of phosphate buffer (pH 7.0). The hemolytic activity eluted as a sharp peak at an ionic strength of 250 mM, and its recovery was found to be 100%. Protein determination indicated that about 75% of protein had been removed (considering the

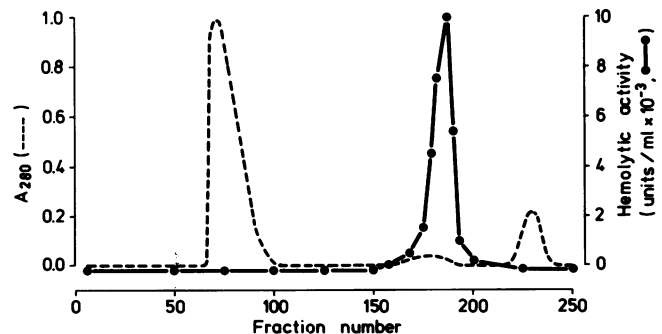


FIG. 1. Fractionation of NMS by gel-permeation chromatography. NMS (35 ml) was loaded on a column (100 by 5.0 cm) packed with Sephadex G-50 and eluted with Tris hydrochloride (100 mM, pH 8.0) in 250 mM NaCl as the effluent buffer. The UV absorbance (A_{280}) was monitored, and the hemolytic activity was measured by the passive hemolysis test as described in Materials and Methods. Fractions of 10 ml were collected.

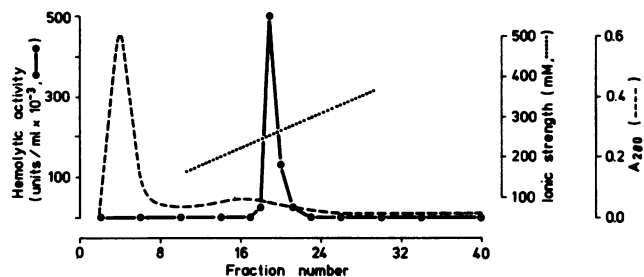


FIG. 2. Purification of factor from NMS by hydroxyapatite chromatography. Factor obtained after G-50 chromatography was bound to hydroxyapatite and eluted with a linear gradient (0 to 500 mM) of phosphate buffer (pH 7.0). UV absorbance (A_{280}) and hemolytic activity were recorded in the effluent. The dotted line represents the slope of the salt gradient.

foregoing preparation as 100%). The elution profile is shown in Fig. 2.

Thus, in a two-step purification, factor was specifically enriched 1,675-fold, and the overall recovery was 33% in terms of hemolytic activity (Table 1).

Identification of factor and determination of its molecular mass by SDS-PAGE. SDS-PAGE after each step of purification showed the specific enrichment of a band with a molecular mass of 28 kDa. After G-50 chromatography (lane A in Fig. 3), many additional bands were visualized in the range of 66 kDa. After hydroxyapatite chromatography (lane C in Fig. 3), besides the 28-kDa band, only one band of 66 kDa was seen. After Western blot transfer, this band stained with anti-mouse albumin antiserum (data not shown). When the gel was loaded with 10 times more material (lane B in Fig. 3), other proteins were observed, but with very low intensity. When individual fractions after G-50 and hydroxyapatite chromatography, respectively, were analyzed for hemolytic activity and by SDS-PAGE, the 28-kDa band was seen most intensely in fractions containing the maximal hemolytic activity (data not shown). Therefore, this band was assumed to represent factor.

Identification of factor was achieved in two different ways. First, NMS was absorbed with SRBC which had been coated with *A. calcoaceticus* lipid A-OH (specific ligand) whereby the hemolytic activity was completely abolished. As a control, another sample of NMS was absorbed in a similar manner with *E. coli* lipid A-OH (nonspecific ligand) to proof the specificity of the absorption. The two absorbed samples and a nonabsorbed sample were separated each on G-50 as described above, and fractions containing the hemolytic activity were pooled (in case of the specifically absorbed

TABLE 1. Purification of factor from NMS serum by chromatography with Sephadex G-50 and hydroxyapatite (HA)

Prepn	Vol (ml)	Total protein ^a (mg)	Total activity ^b (hemolytic U)	Recovery (%)	Sp act (hemolytic U/mg of protein)
NMS	35	2,415	2.87×10^6	100	1.19×10^3
G-50	170	1.81 ^c	8.70×10^5	30	4.81×10^5
HA	3.7	0.47 ^c	9.40×10^5	33	2.0×10^6

^a Determined by the method of Lowry et al. (24).

^b Determined by the microtiter passive hemolysis test with SRBC coated with *A. calcoaceticus* lipid A-OH.

^c Determined by the Lowry assay after precipitation with TCA.

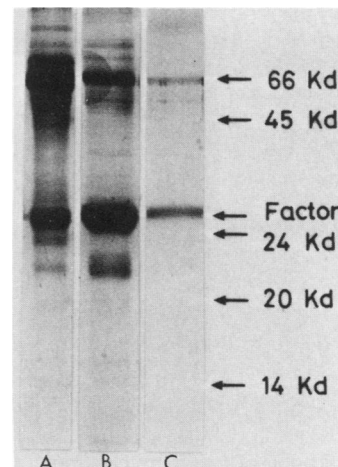


FIG. 3. SDS-PAGE of purified factor from NMS after G-50 (lane A) and hydroxyapatite chromatography (lane C and lane B with 10 times more material). Samples were precipitated with TCA and resolved in sample buffer. Arrows indicate the positions of marker proteins (66 kDa, BSA; 45 kDa, ovalbumin; 24 kDa, trypsinogen; 20 kDa, trypsin inhibitor; 14 kDa, lactalbumin). Electrophoresis conditions were as described by Laemmli (23) with a 14% gel; staining was performed with Coomassie brilliant blue.

sample, corresponding fraction numbers were combined), precipitated with TCA, and subjected to SDS-PAGE (Fig. 4). In all samples, three major bands were visualized with Coomassie blue, one of which exhibiting the same molecular mass as BSA. In addition, the 28-kDa protein was observed in the nonabsorbed sample (lane A in Fig. 4) and in the nonspecifically absorbed sample (lane C); however, it was completely absent in the specifically absorbed sample (lane B).

Second, factor was purified by G-50 chromatography and

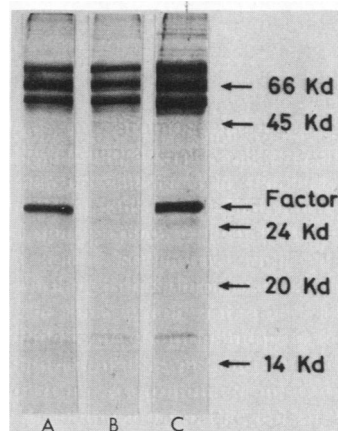


FIG. 4. Identification of factor by absorption. NMS was absorbed with SRBC coated with either *A. calcoaceticus* lipid A-OH (specific ligand) or with *E. coli* lipid A-OH (nonspecific ligand) to exclude nonspecific absorption. Both absorbed samples and a nonabsorbed NMS sample were fractionated by G-50 chromatography (Fig. 1) and analyzed by SDS-PAGE. The band at 28 kDa is seen in the nonabsorbed sample (lane A) and in the nonspecifically absorbed sample (lane C), whereas it is absent in the specifically absorbed sample (lane B). Electrophoresis and staining conditions are as in Fig. 3.

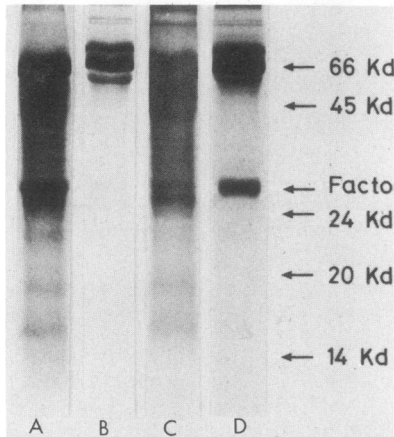


FIG. 5. Identification of factor by absorption. Factor obtained after G-50 chromatography (20 ml) was absorbed with latex particles coated with either the specific ligand (*A. calcoaceticus* lipid A-OH) or a nonspecific ligand (*E. coli* lipid A-OH). After centrifugation, the supernatant (precipitated with TCA) and the sedimented latex particles (boiled in sample buffer) were analyzed by SDS-PAGE. Lanes A and B represent the sediment and the supernatant, respectively, after specific absorption, and lanes C and D represent the sediment and supernatant, respectively, after nonspecific absorption. The 28-kDa band is seen in the sediment after specific absorption (lane A) and in the supernatant after nonspecific absorption (lane D). Electrophoresis and staining conditions are as in Fig. 3.

absorbed with latex particles which had been coated either with the specific ligand (*A. calcoaceticus* lipid A-OH) or the nonspecific ligand (*E. coli* lipid A-OH). After centrifugation, the sedimented particles (boiled in sample buffer) and the supernatant (precipitated with TCA) were analyzed by SDS-PAGE (Fig. 5). When specifically coated latex particles were used, the 28-kDa band was found in the sediment (lane A in Fig. 5), whereas it was absent from the supernatant (lane B). On the other hand, after nonspecific absorption, the band was present in the supernatant (lane D in Fig. 5) and not found on the sedimented latex particles (lane C). Determination of the hemolytic activity after absorption indicated that the absorption was specific and complete (Table 2). Thus, the 28-kDa band was only observed in samples exhibiting hemolytic activity, further supporting our assumption that this protein represents factor.

TABLE 2. Hemolytic activity of NMS and G-50-purified factor before and after absorption

Prepn	Hemolytic activity ^a (U/ml)
NMS (not absorbed)	81,920
Absorbed SRBC- <i>A. calcoaceticus</i> lipid A-OH ^b	100
Absorbed SRBC- <i>E. coli</i> lipid A-OH	81,920
G-50-purified factor ^c (not absorbed)	5,120
Absorbed latex- <i>A. calcoaceticus</i> lipid A-OH ^d	<100
Absorbed latex- <i>E. coli</i> lipid A-OH	5,120

^a Tested with SRBC coated with *A. calcoaceticus* lipid A-OH in the microtiter passive hemolysis test.

^b NMS (1.0 ml) was absorbed with glutaraldehyde-fixed SRBC (50 μ l) to which the respective ligand had been coated (100 μ g per 0.2 ml of packed SRBC).

^c Obtained after chromatography of NMS on Sephadex G-50.

^d Factor (4.0 ml) was absorbed with a suspension of latex beads (100 μ l) coated with respective ligand.

Binding specificity of factor for LPS and isolated core oligosaccharides. The binding of factor to LPS was assayed by the quantitative passive hemolysis inhibition test whereby constant amounts of factor (causing 80% of lysis of the test SRBC) were inhibited by preincubation with various amounts of *A. calcoaceticus* LPS. The reactivity of purified factor was compared to that of unfractionated NMS and to the reactivity of an IgM-rich serum fraction obtained from rabbit immune serum after immunization with heat-killed *A. calcoaceticus* bacteria (Fig. 6). Purified factor and NMS exhibited superimposed sigmoid inhibition curves with a 50% inhibition value of 4 ng of LPS. This suggests that factor alone without additional serum components is able to bind to LPS, followed by activation of guinea pig complement. The inhibition of specific immunoglobulin is also represented by a sigmoid inhibition curve of similar shape; however, 8 times more antigen (64 ng) is required to yield 50% inhibition of lysis. When factor and immune serum were tested by the hemagglutination technique, the latter was found to agglutinate antigen-coated SRBC, whereas the former failed to do so.

The inhibition of purified factor was also tested with isolated oligosaccharides to determine its binding specificity. A di- and trisaccharide with one and two heptose residues, respectively, linked to position 5 of a reducing KDO moiety were prepared from the respective LPS of *S. minnesota* rough mutants (strains R4 and R7) and tested for their inhibition of factor by the passive hemolysis inhibition test (with *A. calcoaceticus* lipid A-OH-coated SRBC as test erythrocytes). Various oligosaccharides (maltose, lactose, raffinose, stachyose, gentiobiose, turanose, palatinose, *N*, *N*-diacetylchitobiose) and the ammonium salt of KDO were tested in parallel (Fig. 7). The trisaccharide α -D-mannoheptopyranosyl-(1 \rightarrow 3)- α -D-mannoheptopyranosyl-(1 \rightarrow 5)-KDO and the disaccharide α -D-mannoheptopyranosyl-(1 \rightarrow 5)-KDO inhibited factor specifically with 50% inhibition values of 30 and 60 μ g, respectively. None of the commercially available oligosaccharides or KDO monosaccharide was able to confer inhibition in concentrations of up to 400 μ g (data not shown).

Inhibition of factor in vivo. To see whether factor binds to LPS under in vivo conditions, C57BL/6 mice were injected

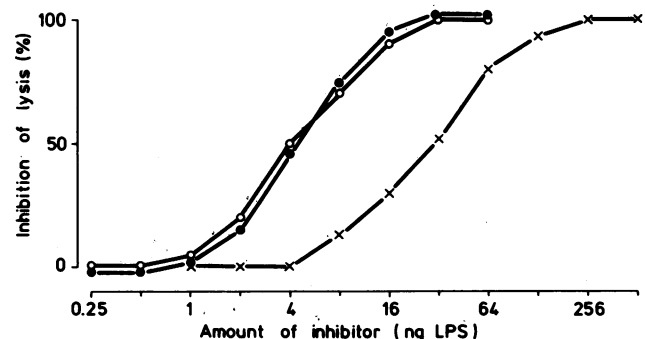


FIG. 6. Inhibition of NMS (●), purified factor after hydroxyapatite chromatography (○), and IgM from rabbit immune serum against *A. calcoaceticus* (×) with *A. calcoaceticus* LPS. Constant amounts of NMS, purified factor, or rabbit hyperimmune IgM causing 80% of lysis of the test erythrocytes were preincubated with various amounts of LPS and tested with SRBC coated with *A. calcoaceticus* lipid A-OH. Inhibition is expressed as the percent loss of hemolytic activity.

with 100 μg of *A. calcoaceticus* LPS intravenously and exsanguinated after various lengths of time (in groups of four), and the hemolytic activity of factor was determined in the serum. The activity came down to zero 5 min after the administration of LPS (Fig. 8). After 2 h, the activity reappeared in the serum (titer of 160) and reached the starting level of 1,280 after 8 h. In another experiment, the injected dose of LPS was varied to determine the minimal amount of LPS necessary for complete inhibition of factor, which was found to be 12 μg of LPS per mouse. With this dose, the time course of factor activity was followed again (Fig. 8). The zero level was reached after 15 min; however, the original level was observed already after 1 h and did not change thereafter over the observed period of time.

Occurrence of factor in mice. Different inbred strains of mice were tested for the presence of factor by determining the hemolytic activity in sera. The following strains were found to exhibit factor activity: C57BL/6, NMRI, BALB/c, CBA/N, ASW/Sn, AJ, C57H2K, CWB, ATH, B10.AQR, A.A.L., C3H/HeJ, C3H/Tif, C3H/HeN, B10.D2, B10.A4R, and BALB/c *nu nu*. Thereby we observed that, within a given mouse strain, no variation at all occurred from one individual to another (groups of six mice were tested), and that interstrain variation was neglectably low, between 1,280 and 5,120. Factor was present in newborn mice (strain C57BL/6), however, with significantly lower activity (titer of 32). In 10-day-old mice, the activity was expressed to the same extent as in adult animals. Immunization of mice (strain NMRI) with heat-killed *A. calcoaceticus* bacteria did not influence the activity of factor, independent of whether the animals were immunized with a single or repeated injections of immunogen.

Physicochemical stability of factor. The stability of factor in whole NMS was followed under different physicochemical conditions with respect to hemolytic activity. Factor was found to be stable at 4°C for at least 1 month and at -20°C for more than 1 year. Factor was also stable for 2 h at 37°C and for 1 h at 45°C; however, it was completely inactivated at 56 and 60°C after 20 and 5 min, respectively. NMS could be treated at 4°C with chloroform or 1,1,2-trichlorotrifluoroethane without loss of activity, whereas treatment with

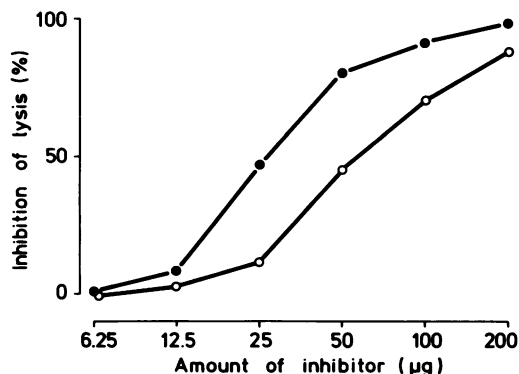


FIG. 7. Inhibition of factor by isolated oligosaccharides from LPS of *S. minnesota* rough mutants. Constant amounts of factor, causing 80% of lysis of the test erythrocytes, were preincubated with various amounts of α -D-mannoheptopyranosyl-(1 \rightarrow 5)-KDO (○) or α -D-mannoheptopyranosyl-(1 \rightarrow 3)- α -D-mannoheptopyranosyl-(1 \rightarrow 5)-KDO (●) and tested for the residual hemolytic activity with SRBC coated with *A. calcoaceticus* lipid A-OH. Inhibition is expressed as the percent loss of hemolytic activity.

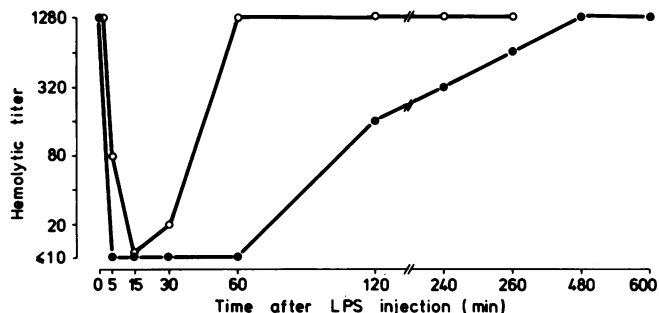


FIG. 8. Inhibition of factor under in vivo conditions. NMRI mice were injected with 100 μg (●) or 12 μg (○) of *A. calcoaceticus* LPS intravenously and exsanguinated after various lengths of time. The hemolytic activity of factor was determined in the serum by using SRBC coated with *A. calcoaceticus* lipid A-OH. Each point represents the mean value obtained from four mice.

2-mercaptoethanol (0.1 M, room temperature for 1 h) or trypsin (0.1%, 37°C for 30 min) abolished the hemolytic activity completely. Purified factor after G-50 and hydroxyapatite chromatography was stored for 3 months at -70°C without loss of hemolytic activity. Factor could be precipitated from NMS with ammonium sulfate at a concentration of 33 to 50% (4°C) or with polyethylene glycol (4 to 10%). However, the purification and the recovery were poorer than with G-50 chromatography as the first step of purification. Factor exhibited hydrophobic properties leading to nonspecific absorption to glass and plastic surfaces and to various gel supports. By this, factor was bound to polyacrylamide (Bio-Gel), to polyacrylamide-agarose gels (Sephacryl), and to controlled porous glass. As shown above, factor was also slightly retained by Sephadex G-50, however, the retention was much less pronounced than with other supports. Also the contact with dialysis tubing resulted in considerable loss of activity; therefore dialysis steps were avoided during the preparation of purified factor.

DISCUSSION

In earlier publications, we have reported on a new type of common antigen of gram-negative bacteria which resides in the so-called inner-core region (heptose-KDO region) of bacterial LPS (4, 5, 12; Brade et al., in press). This antigenic determinant could be differentiated from all yet described antigenic specificities of LPS, e.g., the core (25) and lipid A antigenic determinants (9, 15, 31) by its specific interaction—as measured in a passive hemolysis assay—with rabbit immune serum elicited against heat-killed bacteria of the genus *Acinetobacter*, a member of the *Neisseriaceae* family. In the LPS and lipid A of *Acinetobacter* spp., the antigen was first detected and found to be particularly well exposed. However, we found later that the new type of antigen is present in all LPSs containing KDO and neutral sugars (2, 5). Since the deep rough mutants of the Re chemotype contain only a KDO disaccharide as sugar constituents of the core (7, 8), they did not express the aforementioned antigenic specificity. Immunochemical investigations indicated that the neutral sugar, required in addition to KDO, could be either heptose (as found in all enterobacterial and many other LPSs), glucose, as determined in the LPS of *A. calcoaceticus* (3), or mannose, as shown by Smith degradation of enterobacterial LPS (5). We have also reported earlier, that normal serum of many mammalian species—including humans—contains a naturally occurring activity which binds to the above-mentioned antigenic determinant.

The highest activities were found in mice and rats, where titers of up to 5,120 were observed. The activity was measured by the ability of the serum to lyse antigen-coated SRBC in a complement-dependent assay. The activity was assumed to constitute "natural antibody" (4); however, its immunoglobulin nature had not been proven. In the mean time it became evident that in mice this naturally occurring, LPS-binding factor is not immunoglobulin (unpublished observation). Therefore, we started to investigate the nature and the biological properties of this factor.

Factor was purified from normal serum of NMRI mice by two chromatography steps on Sephadex G-50 and hydroxyapatite, respectively. After these steps, factor was enriched 1,675-fold, and the overall recovery was 30% in terms of hemolytic activity (Table 1). Factor exhibited hydrophobic properties and tended to form aggregates during the purification. Therefore, the hemolytic activity of factor may have been underestimated, and the specific purification may be even more efficient. SDS-PAGE of purified factor under reducing conditions showed two major bands. One band had a molecular mass of 28 kDa; the second migrated to the same position as BSA and stained after Western blot transfer with anti-mouse albumin and thus was regarded as contamination with serum albumin. Since the hemolytic activity of factor could not be assayed after electrophoresis because of its sensitivity to various detergents (data not shown), and, moreover, since an antiserum against factor is not yet available, identification of factor was achieved by absorption experiments. NMS was absorbed with the specific ligand (*A. calcoaceticus* lipid A-OH) and purified in parallel to a nonabsorbed sample. All bands but the 28-kDa protein were visualized after SDS-PAGE in the absorbed sample. The specificity of the absorption was demonstrated by using an antigenically unrelated antigen of similar hydrophobicity (*E. coli* lipid A-OH) which did not abolish factor activity. Since antigen-coated SRBC were used for this experiment, elution of absorbed factor from SRBC was not possible. Therefore, absorption was also carried out with antigen-coated latex particles. To avoid nonspecific binding to the hydrophobic surface of latex beads, the active sites were blocked with BSA. In addition, latex beads coated with an antigen of similar physicochemistry but no binding specificity for factor (*E. coli* lipid A-OH) were used as a control. The sedimented particles (together with bound factor) were boiled in SDS buffer and subjected to SDS-PAGE; the supernatants were analyzed after precipitation with TCA. When specifically coated latex beads were used, the 28-kDa protein was found in the sediment, whereas it was absent from the supernatant. The opposite result was obtained when nonspecifically coated latex particles were used for absorption. Moreover, individual fractions after G-50 and hydroxyapatite chromatography were analyzed by SDS-PAGE, and the intensity of the 28-kDa band had its maximum in the same fraction as the hemolytic activity. From these results we conclude that the 28-kDa protein represents naturally occurring LPS-binding factor in NMS.

In another part of our work, we investigated some biological properties of factor, notably its binding to LPS. We have reported already on numerous LPSs interacting with NMS by means of a passive hemolysis inhibition assay (5). We compared the reactivity of isolated factor to the one of whole NMS, which was not different with respect to binding specificity. In quantitative inhibition studies (Fig. 5), isolated factor and NMS were inhibited by *A. calcoaceticus* LPS to the same extent, exhibiting identical sigmoid inhibition curves and the same 50% inhibition value. However, when

an IgM-rich fraction of rabbit immune serum against the common antigen was tested by the passive hemolysis inhibition test, 8 times more antigen was required to cause 50% inhibition. These results, together with the finding that factor did not agglutinate antigen-coated SRBC (whereas immune serum did so), led us to propose that factor has only one binding site. The region of LPS to which NMS binds has been proposed to contain 5-*O*-glycosylated KDO. Here we have demonstrated that isolated oligosaccharides containing such structures are specific and effective inhibitors of purified factor (Fig. 6).

In another set of experiments, we studied the binding of factor to LPS under in vivo conditions. Mice were injected with *A. calcoaceticus* LPS, and the hemolytic activity of factor was followed kinetically. With 12 μ g of LPS, factor was inhibited completely within the first 15 min and reappeared in the serum shortly thereafter, reaching the starting level after 1 h. Since a de novo synthesis is unlikely in this very short period of time, factor may be released from a nonserum pool, or it may form complexes with LPS which can dissociate in vivo. The very important question of what happens to such complexes cannot be answered now, and experiments are under way to contribute to its answer. The data reported by others concerning the binding of LPS to high-density lipoprotein (13, 36, 37) are not in contradiction to our observation, since in some of these studies (13) nonphysiologically high amounts of LPS (in the milligram range) were used, whereas binding of factor takes place at a concentration which is 1 order of magnitude less (microgram range).

In the literature, several factors have been described that bind to surface structures of gram-positive and gram-negative bacteria (1, 10, 30, 33, 34, 38; S. Lebbar, J. M. Cavillon, M. Caroff, A. Ledur, H. Brade, R. Sarfati, and N. Haeffner-Cavillon, *Eur. J. Immunol.*, in press). Some of them have been investigated carefully, as are the C-reactive protein and the so-called acute-phase proteins (11, 28, 29, 35). We paid special attention to the extensive work of Kawakami et al. describing a naturally occurring bactericidal factor in mammalian species (19-22). Very recently, these authors showed that this factor has been conserved for more than 300 million years during evolution (21). Although there exist striking similarities of the reported data to ours, e.g., the widespread occurrence in animal sera, the inability to be induced by immunization, the sensitivity to heat and mercaptoethanol, and finally the presence of a polypeptide chain with a molecular mass of 28 kDa (20), the factor described herein is different from that reported by Kawakami and co-workers. The bactericidal factor has a highly restricted reactivity which binds to *N*-acetyl-D-glucosamine in LPSs of the Ra core type, whereas our factor recognizes the KDO region present in all LPSs tested so far, except those belonging to the Re chemotype. Therefore, our factor binds to LPS of various gram-negative bacteria such as the *Enterobacteriaceae*, *Neisseriaceae*, *Vibrionaceae*, *Pseudomonadaceae*, and *Bacteroidaceae* (5). For the moment, little is known about the possible biological relevance of this factor, but it seems unlikely that factor is acting on the classical endotoxic activities of LPS, as are the lethal toxicity, the pyrogenicity, or the Shwartzman phenomenon, since these activities are equally expressed by LPS and the polysaccharide-deprived lipid moiety (14, 26, 31, 32). In addition, the LPS of *A. calcoaceticus*, which binds particularly well to factor, is of the same endotoxic potency as the enterobacterial LPSs (6).

In this context, we refer to the results reported by

Haeffner-Cavaillon et al., describing a receptor on macrophages which binds to the polysaccharide region of LPS from *Bordetella pertussis* (18) and activates macrophages to the production of interleukin 1 (17). Moreover, they reported that other LPSs (from *Neisseriaceae* and *Enterobacteriaceae*) are able to inhibit the binding of *B. pertussis* polysaccharide to the macrophage receptor. In addition, they found that serum components other than complement or immunoglobulin are required for optimal binding (16). In addition, the activation of macrophages to secrete interleukin 1 is mediated by the same structure which is recognized by the serum factor described herein: the heptosyl-KDO region of bacterial LPS (Lebbar et al., in press).

Therefore, we speculate that our factor contributes to the binding of LPS to macrophages by a factor-specific receptor or that factor itself is a surface structure of macrophages.

ACKNOWLEDGMENTS

We thank E. T. Rietschel for his encouraging interest in this work and M. Koel and T. Lüderitz for fruitful discussion and criticism. The expert technical assistance of U. Albert and V. Susott is gratefully acknowledged.

This work was supported by the Deutsche Forschungsgemeinschaft (Br 731/3).

LITERATURE CITED

1. Abdelnoor, A. M., N. R. Harvie, and A. G. Johnson. 1982. Neutralization of bacteria- and endotoxin-induced hypotension by lipoprotein-free human serum. *Infect. Immun.* **38**:157-161.
2. Brade, H. 1985. The occurrence of 2-keto-3-deoxyoctonic acid 5-phosphate in lipopolysaccharides of *Vibrio cholerae* Ogawa and Inaba. *J. Bacteriol.* **161**:795-798.
3. Brade, H., and C. Galanos. 1982. Isolation, purification, and chemical analysis of the lipopolysaccharide and lipid A of *Acinetobacter calcoaceticus* NCTC 10305. *Eur. J. Biochem.* **122**:233-237.
4. Brade, H., and C. Galanos. 1983. A new lipopolysaccharide antigen identified in *Acinetobacter calcoaceticus*: occurrence of widespread natural antibody. *J. Med. Microbiol.* **16**:203-210.
5. Brade, H., and C. Galanos. 1983. Common lipopolysaccharide specificity: a new type of antigen residing in the inner core region of S- and R-form lipopolysaccharides from different families of gram-negative bacteria. *Infect. Immun.* **42**:250-256.
6. Brade, H., and C. Galanos. 1983. Biological activities of the lipopolysaccharide and lipid A from *Acinetobacter calcoaceticus*. *J. Med. Microbiol.* **16**:211-214.
7. Brade, H., C. Galanos, and O. Lüderitz. 1983. Isolation of a 3-deoxy-D-mannoctulosonic acid disaccharide from *Salmonella minnesota* rough-form lipopolysaccharides. *Eur. J. Biochem.* **131**:201-203.
8. Brade, H., and E. T. Rietschel. 1984. α -2-4-Interlinked 3-deoxy-D-mannoctulosonic acid disaccharide. A common constituent of enterobacterial lipopolysaccharides. *Eur. J. Biochem.* **145**:231-236.
9. Brade, L., and H. Brade. 1985. Characterization of two different antibody specificities recognizing distinct antigenic determinants in free lipid A of *Escherichia coli*. *Infect. Immun.* **48**:776-781.
10. Chedid, L., M. Parant, F. Parant, and F. Boyer. 1968. A proposed mechanism for natural immunity to enterobacterial pathogens. *J. Immunol.* **100**:292-301.
11. Claus, D. R., J. Siegel, K. Petras, A. P. Osmand, and H. Gewurz. 1977. Interaction of C-reactive protein with the first component of human complement. *J. Immunol.* **119**:187-192.
12. Dröge, W., V. Lehmann, O. Lüderitz, and O. Westphal. 1970. Structural investigations on the 2-keto-3-deoxyoctonate region of lipopolysaccharides. *Eur. J. Biochem.* **14**:175-184.
13. Freudenberg, M. A., T. C. Bog-Hansen, U. Back, and C. Galanos. 1980. Interaction of lipopolysaccharides with plasma high-density lipoprotein in rats. *Infect. Immun.* **28**:373-380.
14. Galanos, C., O. Lüderitz, E. T. Rietschel, O. Westphal, H. Brade, L. Brade, M. Freudenberg, U. Schade, M. Imoto, H. Yoshimura, S. Kusumoto, and T. Shiba. 1984. Synthetic and natural *Escherichia coli* free lipid A express identical endotoxic activities. *Eur. J. Biochem.* **148**:1-5.
15. 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**:115-122.
16. Haeffner-Cavaillon, N., J.-M. Cavaillon, M. Etievant, S. Lebbar, and L. Szabo. 1985. Specific binding of endotoxin to human monocytes and mouse macrophages: serum requirements. *Cell. Immunol.* **91**:119-131.
17. Haeffner-Cavaillon, N., J.-M. Cavaillon, M. Moreau, and L. Szabo. 1984. Interleukin 1 secretion by human monocytes stimulated by the isolated polysaccharide region of the *Bordetella pertussis* endotoxin. *Mol. Immunol.* **21**:389-395.
18. Haeffner-Cavaillon, N., R. Chaby, J.-M. Cavaillon, and L. Szabo. 1982. Lipopolysaccharide receptor on rabbit peritoneal macrophages. I. Binding characteristics. *J. Immunol.* **128**:1950-1954.
19. Ihara, I., Y. Harada, S. Ihara, and M. Kawakami. 1982. A new complement-dependent bactericidal factor found in nonimmune mouse sera: specific binding to polysaccharide of Ra chemotype *Salmonella*. *J. Immunol.* **128**:1256-1260.
20. Ihara, I., H. Ueda, A. Suzuki, and M. Kawakami. 1982. Physicochemical properties of a new bactericidal factor. *Biochem. Biophys. Res. Commun.* **107**:1185-1190.
21. Kawakami, M., I. Ihara, S. Ihara, A. Suzuki, and K. Fukui. 1984. A group of bactericidal factors conserved by vertebrates for more than 300 million years. *J. Immunol.* **132**:2578-2581.
22. Kawakami, M., I. Ihara, A. Suzuki, and Y. Harada. 1982. Properties of new complement-dependent bactericidal factor specific for Ra chemotype *Salmonella* in sera of conventional and germ-free mice. *J. Immunol.* **129**:2198-2201.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
25. Lüderitz, O., C. Galanos, H. J. Risse, E. Ruschmann, S. Schlecht, G. Schmidt, H. Schulte-Holthausen, R. Wheat, O. Westphal, and J. Schlosshardt. 1966. Structural relationships of *Salmonella* O and R antigens. *Ann. N.Y. Acad. Sci.* **133**:349-374.
26. Lüderitz, O., K. Tanamoto, C. Galanos, G. R. McKenzie, H. Brade, U. Zähringer, E. Th. Rietschel, S. Kusumoto, and T. Shiba. 1984. Lipopolysaccharides: structural principles and biologic activities. *Rev. Infect. Dis.* **6**:428-431.
27. Neter, E. 1956. Bacterial hemagglutination and hemolysis. *Bacteriol. Rev.* **20**:166-188.
28. Oliveria, E. B., E. Gotschich, and T.-Y. Liu. 1980. Comparative studies on the binding properties of human and rabbit C-reactive proteins. *J. Immunol.* **124**:1396-1402.
29. Osmand, A. P., R. F. Mortensen, J. Siegel, and H. Gewurz. 1975. Interaction of C-reactive protein with the complement system. III. Complement-dependent passive hemolysis initiated by CRP. *J. Exp. Med.* **142**:1065-1077.
30. Ribí, E., J. L. Cantrell, K. Takayama, N. Qureshi, J. Peterson, and H. O. Ribí. 1984. Lipid A and immunotherapy. *Rev. Infect. Dis.* **6**:567-572.
31. 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.
32. Rietschel, E. T., H. Mayer, H.-W. Wollenweber, U. Zähringer, O. Lüderitz, O. Westphal, and H. Brade. 1984. Bacterial lipopolysaccharides and their lipid A component, p. 11-22. *In* J. Y. Homma, S. Kanegasaki, O. Lüderitz, T. Shiba, and O. Westphal (ed.), *Bacterial endotoxin*. Chemical, biological and clinical aspects. Verlag Chemie, Weinheim.
33. Rowley, D. 1968. Sensitivities of rough gram-negative bacteria

- to the bactericidal action of serum. *J. Bacteriol.* **95**:1647-1650.
34. **Rudbach, J. A., and A. G. Johnson.** 1962. Changes in serologic reactivity of endotoxin induced by fraction IV (Cohn) of normal human serum. *Proc. Soc. Exp. Biol. Med.* **111**:651-655.
35. **Tobias, P. D., K. P. W. H. McAdam, and R. J. Ulevitch.** 1982. Interactions of bacterial lipopolysaccharide with acute phase rabbit serum and isolation of two forms of serum amyloid A. *J. Immunol.* **128**:1420-1427.
36. **Ulevitch, R. J., A. R. Johnston, and D. B. Weinstein.** 1979. New function for high density lipoproteins. *J. Clin. Invest.* **64**:1516-1524.
37. **Ulevitch, R. J., A. R. Johnston, and D. B. Weinstein.** 1981. New function for high density lipoproteins (HDL). II. Isolation and characterization of a bacterial lipopolysaccharide-HDL complex formed in rabbit plasma. *J. Clin. Invest.* **67**:827-837.
38. **Yoshioka, M., and A. G. Johnson.** 1962. Characteristics of endotoxin altering fractions derived from normal human serum. *J. Immunol.* **89**:326-335.