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Human Complement Activation by Lipopolysaccharides from *Bacteroides oralis, Fusobacterium nucleatum,* and *Veillonella parvula*

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The properties of different lipopolysaccharide (LPS) preparations to induce C3 conversion in human serum was studied by means of crossed immunoelectrophoresis. C3 conversion by the alternative pathway was evaluated after calcium depletion, and lipid A-dependent activation was measured by means of inhibition with polymyxin B sulfate. LPS from *Bacteroides oralis* converted C3 mainly via the alternative pathway, whereas LPS from *Fusobacterium nucleatum* and *Veillonella parvula* converted C3 mainly by the classical pathway. *Veillonella* LPS caused the most pronounced lipid A-dependent conversion. The results are discussed in relation to the chemical composition of the LPS preparations.

Lipopolysaccharides (LPS) from the cell wall of gram-negative bacteria have for many years been known to induce consumption of complement, preferentially factors C3 through C9 (9), with subsequent inflammatory response of the host (16). However, Galanos et al. (8) showed that LPS from different bacterial strains interact in qualitatively different ways with complement. Some preparations did not consume complement at all, although the isolated lipid A portion interacted with complement (8), and this fact indicated that lipid A could be the part of the LPS complex that was responsible for the complement activation. Several reports (6, 7, 20) have shown that LPS may activate complement by both the classical and the alternative pathways, and recently it has been stated that the lipid A portion of LPS activates complement by the classical pathway, whereas the polysaccharide portion activates the complement system by the alternative pathway (20). The pathophysicological changes, resulting from interaction of LPS with complement, differ between LPS conversion by the classical and alternative pathways (7, 21).

The anaerobic gram-negative bacteria have gathered increasing interest as possible pathogenic organisms in many infections (1). These bacteria are frequently found in association with many oral infections, e.g., in periodontal disease (26) and periapical inflammation of teeth (G. Sundqvist, Ph.D. thesis, Umea University, Umea, Sweden, 1976), in which endotoxin may participate as a pathogenetic factor. From this background, we addressed ourselves to the activation of complement by LPS from three anaerobic bacteria to investigate their ability to activate complement by the classical and alternative pathways.

MATERIALS AND METHODS

Bacterial strains. *Bacteroides oralis* Bact-MC3, *Fusobacterium nucleatum* Fus-MC8, originally isolated from the root canal of a monkey tooth, and *Veillonella parvula* Veill-H3, isolated from human dental plaque, were used.

Cultivation. The culture medium used for cultivation of Bact-MC3 and Fus-MC8 was that described by Dahlén and Hofstad (4). Cultivation was carried out in a fermentor with stirring at a constant pH of 7.2. The cells were harvested at the end of the logarithmic phase. Cultivation of Veill-H3 was performed in a medium, containing proteose peptone (Difco 0122-01), 40 g; $Na_2HPO_4 \cdot 2H_2O$, 1.0 g; KH_2PO_4, 0.4 g, sodium lactate 50%, 25 ml, and re-distilled water, 965 ml. After sterilization, 10 ml of a salt stock solution made by the method of Linder et al. (15) was added.

Isolation of LPS. LPS was extracted with 45% phenol for 15 min at room temperature (28) and further prepared as described elsewhere (5). Stock solutions of LPS (0.5 or 1 mg/ml) were prepared by sonication in phosphate-buffered saline (PBS). The LPS preparations from the different strains are abbreviated Bact-LPS, Fus-LPS, and Veill-LPS, respectively.

Alkaline hydrolysis of LPS. Alkaline hydrolysis of LPS was performed as modified from Neter et al. (22). LPS (5 mg) was dissolved in 1 ml of 1 N NaOH and heated for 1 h, 1 ml of 1 N HCl was added, and the solution was diluted with distilled water to give a final LPS concentration of 1 mg/ml.

Chemical analysis. The procedures for paper chromatography and quantitative estimations for pro-

tein, neutral sugars, fatty acid esters, hexosamine, and ketodeoxyoctonate (KDO) content were carried out as described by Dahlén and Hofstad (4).

Preparation of IC and zymosan. Immune complexes (IC) were obtained by mixing rabbit anti-human serum albumin and human serum albumin (Sigma Chemical Co., St. Louis, Mo.). The preparations were stored at room temperature for 1 to 3 h before use. Zymosan (Sigma) was suspended in PBS at a concentration of 5 mg/ml.

Preparation of PMB-LPS. PMB-LPS was prepared by the procedure described by Morrison and Jacobs (19), slightly modified. Polymyxin B sulfate (PMB) (Sigma) was dissolved (2 mg/ml) in pyrogen-free distilled water and allowed to react with LPS (0.5 mg of PMB to 1 mg of LPS) for 2 h at room temperature. The PMB-LPS stock solution was freshly prepared before serum incubations.

Absorption of serum. Test sera containing antibodies to Veill-LPS were absorbed with alkali-hydrolyzed Veill-LPS for 1 h at 4°C. The precipitates were removed by centrifugation at 5000 × *g* for 20 min at 4°C.

Preparation of EGTA-serum and EDTA-serum. Ethylene glycol-(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) (Sigma) was dissolved in 1 N NaOH and adjusted to 0.1 M, pH 7.4. Magnesium sulfate was added to give a magnesium ion concentration of 0.02 M (24). The EGTA solution was diluted with 9 parts of test serum 10 min before the incubations (see below). Ethylenediaminetetraacetic acid (EDTA) was dissolved in distilled water. The pH was adjusted to 7.4 with 1 N NaOH, and the volume was adjusted to give a 0.1 M solution. The stock solution was diluted with 9 parts of test serum 10 min before incubations (see below).

Serum incubations. Fresh human blood was obtained from three healthy males (age 30 to 50 years) and was allowed to clot at 4°C. Serum was prepared by centrifugation within 1 h and was stored frozen for 1 to 5 days before experiments. A 45-μl amount of serum, serum-EGTA, or serum-EDTA was incubated at 37°C for 1 h with 5 μl of PBS, with or without LPS, PMB-LPS, IC, or zymosan. The final concentrations of LPS, PMB-LPS, and IC were 10 or 100 μg/ml, whereas zymosan was tested at a final concentration of 500 μg/ml. The incubation time was varied from 15 min to 4 h in one series of experiments designed to determine the optimal reaction time. Based on these results 1 h was consequently used. Serum with PBS, serum-EGTA, serum-EDTA, serum + PMB, and zymosan-PMB were used as controls. Experimental variations were tested in experiments performed simultaneously.

C3 conversion assay. Crossed immunoelectrophoresis (IE) of the method of Laurell (14) was performed as described elsewhere (23). Electrophoresis conditions were, briefly, as follows. The material was separated in 1% agarose (Litex, Glostrup, Denmark), dissolved in barbital buffer (pH 8.6; ionic strength, 0.05 M), containing 1 mM calcium lactate. The first electrophoretic separation step was performed for 80 min with a potential drop of 5 V/cm, whereas the second electrophoretic separation was performed for about 18 h at 2 V/cm. A rabbit anti-human C3 antiserum (batch 10-062-084 Dako, Copenhagen, Denmark) (final antiserum dilution, 1:40) was incorporated in the gel during the second run. After electrophoresis unprecipitated protein was eluted, and the plates were dried and stained with Coomassie brilliant blue R.

Quantitation of C3 conversion. The rockets, formed after crossed IE of the test serum against anti-human C3, were approximated to triangles, the surfaces of which were calculated. The area of the anodal triangle in percentage of the total surface was used as a quantitative index of C3 conversion. Experiments with high blank values of serum and PBS were not regarded as reliable for evaluation. Pilot experiments were performed to determine the amount of each of the LPS preparations causing a conversion index of about 50% (Fig. 1 and 2) to make observation of changes easier. A 100-μg/ml concentration of LPS of each of the three preparations was found to be satisfactory for further experiments.

Serological methods. Indirect hemagglutination was performed as described by Hofstad (12).

RESULTS

Chemical analysis of LPS. The results of the chemical analysis of LPS from Bact-MC3, Fus-MC8 (5), and Veill-H3 are given in Table 1. Bact-LPS had a high relative sugar content, mainly glucose, but contained only a small amount of lipid. Veill-LPS had high relative amount of lipid, whereas Fus-LPS had almost equally high relative amounts of lipid and sugar.

C3 conversion. The conversion of C3 in different sera induced by LPS, IC, or zymosan is shown in Fig. 1 and in Table 2. The values of the conversion indexes are means of values obtained from different experiments. The mean conversion index for IC was 67%. Bact-LPS had a somewhat lower reactivity (29%) than Fus-LPS and Veill-LPS (35 and 40%, respectively). The corresponding value for zymosan was 49%. The conversion index for Veill-LPS in absorbed serum was 54%.

Conversion of C3 after magnesium or calcium depletion, or both. The ability of the different preparations to convert C3 in the presence of EGTA or EDTA was measured in another series of experiments, and the results are shown in Fig. 1d and 2a to d and in Table 2. EDTA treatment of serum resulted in complete inhibition of the C3 conversion induced by LPS, IC, and zymosan. In the presence of EGTA, the C3 converting ability of the different LPS preparations, IC, and zymosan was also substantially reduced, although to a varying extent. No conversion was induced by Veill-LPS in the presence of EGTA (Fig. 2d), whereas the conversion values for Fus-LPS and IC were higher than the spontaneously occurring conversion obtained in the control tubes (Fig. 2b and c; Table 2). The conversion induced by Bact-LPS was least affected by the calcium depletion (Fig. 2c), the

TABLE 1. *Results from chemical analysis of LPS*[a]

LPS	Protein	Neutral sugar	Fatty acid esters	Hexosamine	KDO	Main sugars	Other sugars in trace amounts
Bact-MC3	2.6	57.6	5.5	2.0		Glucose	Glucosamine Galactose Rhamnose Ribose
Fus-MC8	5.9	29.7	22.7	6.3	2.0	Heptose Glucose Glucosamine Rhamnose	
Veill-H3	1.7	17.2	18.0	2.2	2.0	Glucose Galactose Glucosamine Galactosamine	

[a] Values are given in percentage of dry weight.

conversion index amounting to 11%. Zymosan, finally (Fig. 2a), gave a conversion index of 24% in the presence of EGTA. Significant antibody titer (1:50) against Veill-LPS was found in the test serum.

Conversion of C3 by PMB-LPS and alkali-treated LPS. The reduction in C3 converting ability after PMB treatment of LPS, as compared to that of native LPS, is shown in Fig. 3 and in Table 3. PMB reduced the ability of Bact-LPS and Fus-LPS to convert C3 to about 10%, as compared to the parallel run with native LPS. The conversion by Veill-LPS was reduced with 46%. PMB treatment did not alter the C3 converting ability of zymosan or IC, and PMB alone did not induce C3 conversion in controls. The test serum contained antibodies to Veill-LPS. In serum depleted of antibodies, PMB reduced the C3 converting ability of Veill-LPS with 32%. Alkali treatment of LPS (22) reduced its ability to convert C3. The converting ability of Veill-LPS was reduced with 80%, whereas the converting ability of Bact-LPS and Fus-LPS was reduced to a less extent (29 and 16%, respectively) (Table 3).

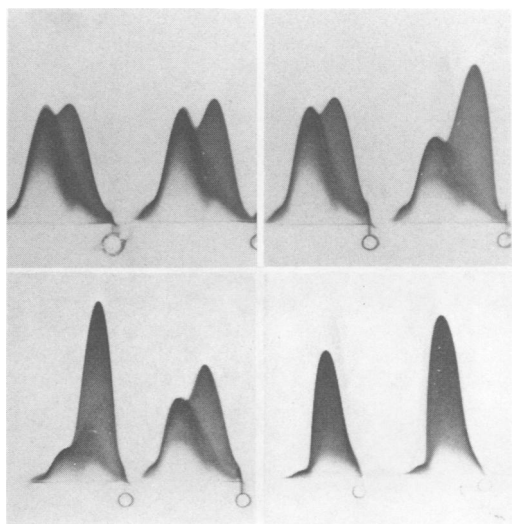

FIG. 1. *Crossed IE against anti-human C3. The test serum was incubated with: (a) (left) Zymosan; (right) IC (100 μg/ml); (b) (left) LPS from Fusobacterium (100 μg/ml); (right) LPS from Bacteroides (100 μg/ml); (c) (left) PBS; (right) LPS from Veillonella (100 μg/ml); (d) (left) IC in EDTA-serum; (right) PBS in EDTA-serum.*

DISCUSSION

Reactions involving complement can be measured as, for example, complement consumption, complement inactivation or C3 conversion. In the present study, a C3 conversion assay was used (14). The conversion index, expressed as the area of the rocket formed by converted C3 in percentage of the total area, is not assumed to be linear to the concentration of inducers, i.e., LPS, IC, and zymosan, or inhibitors, i.e., EGTA and PMB. These pitfalls were overcome by performing experimental variations in simultaneous runs (see figures) and by trying to keep conversion indexes at comparable levels in the experiments. The index values, shown in the tables, should thus be regarded as semiquantitative values, indicating the statistical variability of the method. Conversion indexes are comparable only when compared to values obtained from experiments with equal concentrations of inducers or inhibitors. The results are discussed in qualitative rather than in quantitative terms.

Induction of C3 conversion by the alternative pathway was studied by depletion of calcium ions from the serum by the selective chelator EGTA (3) in the presence of an optimal concentration of magnesium ions (24). Conversion caused by interaction between lipid A and the

TABLE 2. *Influence of ion chelating agents on the C3 conversion induced by IC, zymosan, and LPS extracted from B. oralis, F. nucleatum, and V. parvula*[a]

Determination	Native serum		Ca^{2+}-depleted serum (EGTA)		Ca^{2+}- and Mg^{2+}-depleted serum (EDTA)		Veill-LPS absorbed serum	
	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean	*n*	Mean	*n*
Bact-LPS	29 ± 4.6	5	11 ± 1.1	5	ND[b]			
Fus-LPS	35 ± 2.2	4	3 ± 0.6	4	ND			
Veill-LPS	40 ± 3.0	4	1 ± 0.3	4	<1	4	54	1
IC	67 ± 7.2	2	3 ± 1.1	2	<1	4		
Zymosan	49	1	24	1	<1	1		
Serum control	5 ± 0.9	5	1 ± 0.22	5	<1	1		
Serum + PMB	4	1						

[a] Conversion expressed as conversion index (see text). Mean values ± standard error of the means (SEM) calculated from the results of the indicated number (*n*) of experiments.
[b] ND, Not done.

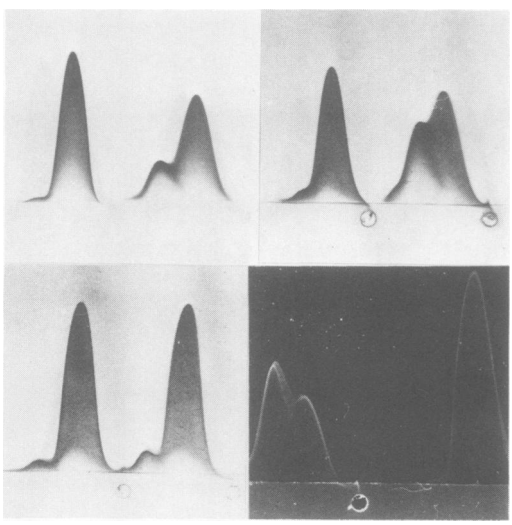

FIG. 2. *Crossed IE as in Fig. 1. The test serum was incubated with: (a) (left) Zymosan in EDTA-serum; (right) Zymosan in EGTA-serum; (b) (left) IC in EGTA-serum; (right) IC in serum; (c) (left) LPS from Fusobacterium in EGTA-serum; (right) LPS from Bacteroides in EGTA-serum. (d) Picture taken of immuno-precipitates before staining with Coomassie brilliant blue R. (left) LPS from Veillonella in serum; (right) LPS from Veillonella in EGTA-serum*

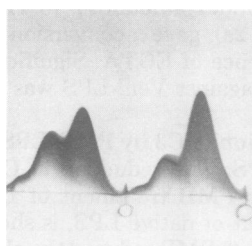

FIG. 3. *Crossed IE as in Fig. 1 and 2. (left) LPS from Veillonella in serum; (right) PMB-LPS from Veillonella in serum.*

TABLE 3. *Influence of PMB and alkali hydrolysis on the C3 conversion induced by LPS from B. oralis, F. nucleatum, and V. parvula*[a]

Determination	PMB		Alkali-hydrolyzed LPS	
	Mean ± SEM	*n*	Mean	*n*
Bact-LPS	14 ± 3.2	3	29	1
Fus-LPS	8 ± 1.9	3	16	1
Veill-LPS	46 ± 4.9	3	80	1
IC	0	1		
Zymosan	0	1		
Beill-LPS-absorbed serum	32	1		

[a] Values are percent reduction. SEM, Standard error of the mean; *n*, number of experiments.

complement factors C1 to C4 was studied by comparing the conversion ratios caused by LPS and PMB-LPS (19) or alkali-treated LPS (22). PMB has been shown to abolish lipid A-dependent complement conversion (18, 19), and the reduction in conversion which occurs after PMB treatment of LPS, as compared to native LPS, may consequently be considered to be an expression of lipid A-dependent conversion. Alkali treatment of LPS reduces its lipid A-dependent activity (22) and was used together with PMB treatment to obtain two independent indications of lipid A-dependent activity.

IC is regarded as an activator of complement mainly by the classical pathway. In the present study, IC was shown to cause a slight conversion by the alternative pathway as well (Table 2). The C3 conversion via the alternative pathway is, however, negligible when compared to IC-

induced conversion in the presence of calcium ions (classical pathway) and could be caused by either a conversion induced by, e.g., aggregated anti-human serum albumin antibodies of the immunoglobulin A type (10) or by endotoxin, present as a contamination in the human serum albumin (2). LPS from *Bacteriodes fragilis* has been shown to convert guinea pig complement in the absence of C4, i.e., by the alternative pathway (27). In the present study, LPS from *B. oralis* converted human complement by the alternative pathway. The reduction of conversion in the absence of calcium as compared to the values obtained in the presence of calcium was about 60%. The same phenomenon is observed with zymosan. In this instance calcium depletion causes a 50% reduction of conversion (Table 2). This is consistent with the findings made by Snyderman and Pike (25), who, using a hemolytic assay, showed that the calcium depletion reduces complement conversion with 40 to 60%. LPS from *Bacteroides* and *Fusobacterium* have little lipid A activity, as PMB treatment and alkali treatment decreased conversion to a limited extent. This should be compared with the extensive reduction obtained when Veill-LPS was treated with PMB or alkali. The conversion by the alternative pathway, induced by LPS from *Bacteroides,* is well in accordance with the suggestions of Morrison and Kline (20) that the polysaccharide portion of LPS induces the conversion via the alternative pathway. Bact-LPS was shown to have a substantially higher content of neutral sugars, notably glucose rather than Fus-LPS and Veill-LPS, whereas its content of lipid was lower than that of the other two.

Gewurz et al. (9) have shown that LPS from *Veillonella* consume preferentially the complement factors C3 through C9. However, in serum depleted of C4 with shark serum factor (13), neither LPS nor IC consumed complement (16). Later, using LPS from *Salmonella typhosa,* the same authors (17) showed that minimal amounts of C1 to C4 were necessary to induce complement consumption by this LPS preparation, although the consumption profile was preferentially C3 through C9. Thus, the consumption profile found (9) was not necessarily due to an activation via the alternative pathway, a fact that was stressed by the authors. In the present study no C3 conversion was induced by Veill-LPS in the presence of EGTA (Fig. 2d). This would imply that activation occurred only via the classical pathway. The serum used for the conversion assays contained a significant amount of antibodies against Veill-LPS which could contribute to this conversion. However, more probably, the activation via the classical pathway is an induction by lipid A. The importance of lipid A-induced activation is strongly indicated by the fact that Veill-LPS converted C3 in serum depleted of antibodies as well (Table 2), and the conversion of C3 in both native and absorbed serum was inhibited by PBM treatment or alkali treatment of LPS (Fig. 3, Table 3). Furthermore, PMB did not alter the C3 converting ability of IC or zymosan; the PMB-induced inhibition of C3 conversion is thus specific for LPS.

The results also agree with the results of the chemical analysis (cf. Table 1) which showed that Veill-LPS had a relatively high content of lipids and a comparatively small amount of neutral sugars.

Whole cells of *F. necrophorum* have been shown to activate C4-deficient guinea pig serum, i.e., via the alternative pathway (11). In the present study, LPS from *F. nucleatum* was shown to convert C3 by both pathways. The conversion by the alternative pathway (Fig. 2c, Table 2) was only slightly above controls and the reduction in conversion by the classical pathway after treatment with PMB. Alkali treatment of LPS shows that Fus-LPS has a low lipid A activity. The low conversion via the alternative pathway, as compared to Bact-LPS, may indicate that Fus-LPS has short polysaccharide chains, whereas those of Bact-LPS would be longer (20). The low decrease in conversion after PMB treatment might indicate that lipid A is not so much exposed in Fus-LPS as in Veill-LPS, although the total lipid content in Fus-LPS is higher.

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