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Antibody-Independent Activation of the Classical Pathway of Human Serum Complement by Lipid A Is Restricted to Re-Chemotype Lipopolysaccharide and Purified Lipid A

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Incubation of most bacterial lipopolysaccharides (LPS) with normal human sera at 37°C activates the serum complement system, resulting in decreased levels of hemolytic complement. A panel of R-chemotype LPS preparations isolated from *Salmonella minnesota* rough mutant strains, as well as smooth wild-type LPS from *S. minnesota*, *Escherichia coli* O55-B5, *Serratia marcescens*, and *Yersinia enterolitica*, were used to examine the effect of LPS polysaccharide chain length on LPS lipid (lipid A)-dependent activation of the classical pathway of complement (CPC). To examine specific lipid A-dependent activation of the CPC, sera deficient in alternative pathway of complement activity were prepared by the removal of factor D. Absorption of normal human sera with formalinized rabbit erythrocytes was found to remove natural antibodies, factors capable of forming LPS complexes which activate the CPC, or both. By using such factor D-depleted formalinized rabbit erythrocyte-absorbed normal human sera, only isolated lipid A and Re-chemotype LPS (R595 LPS) were found to activate the CPC. Thus, the presence of the additional monosaccharide L-glycero-D-mannoheptose in the Rd_2 LPS oligosaccharide chain compared with the L-glycero-D-mannoheptose-deficient Re-chemotype LPS structure is sufficient to block lipid A-dependent activation of the CPC by LPS.

Bacterial lipopolysaccharides (LPSs) have been shown to activate both the classical and alternative pathways of the serum complement system (CPC and APC, respectively) by mechanisms which do not require the participation of anti-LPS antibody (18). In contrast, the lack of APC activation by either purified lipid A or LPS from deep rough mutants (e.g., Re) suggests an obligate requirement for the presence of LPS polysaccharide for LPS-mediated activation of the APC (10, 18, 22). LPS-mediated activation of the CPC is dependent on structural features embodied in the lipid region (lipid A) of the LPS molecule (5).

LPS preparations from several bacterial species have been reported to activate the CPC (15, 18). However, because of the presence of numerous immunoglobulin (6, 32) and nonimmunoglobulin (1, 13, 27, 30) serum proteins which may bind LPS polysaccharide determinants, it is not clear whether LPS preparations directly activate the CPC via a lipid A-mediated activation of the CPC component C1. While some of these LPS-binding proteins promote the activation of the complement system (1, 13), the effects of other LPS-binding proteins on LPS-mediated complement activation are not known. To characterize the influence of relative LPS polysaccharide chain length on lipid A-mediated CPC activation, a panel of R-chemotype LPS preparations from *Salmonella minnesota* were examined for their capacity to activate the CPC. For these experiments APC-deficient (D^-) normal human serum (NHS) was prepared by the removal of factor D and absorbed with formalinized rabbit erythrocytes (f-RRBCs) to remove natural anti-rabbit erythrocyte (anti-RBBC) antibodies present in serum (9, 23). Because some of these natural antibodies are specific for α-galactosyl residues (9) and concanavalin A has been reported to bind both rabbit erythrocytes (RRBCs) (26) and *S. minnesota* LPS (17), I reasoned that this treatment might remove several LPS cross-reactive proteins from serum. In this study I demonstrate that LPS preparations with polysaccharide structures greater in size than the deep rough (Re) chemotype do not activate the CPC in a lipid A-dependent manner.

MATERIALS AND METHODS

LPS. LPS from the Re mutant *S. minnesota* R595 was extracted by the phenol-chloroform-petroleum ether (PCP) method described by Galanos et al. (8). Smooth LPSs from *Escherichia coli* O55:B5, *Yersinia enterocolitica*, and *Serratia marcescens* were prepared by extraction with hot phenol-water, as described by Westphal et al. (29), followed by chromatography on Sepharose 4B (Pharmacia Diagnostics, Piscataway, NJ) (19). Phenol-extracted and Sepharose 4B-chromatographed smooth LPSs from *S. minnesota* wild-type bacteria and PCP-extracted rough LPS from *S. minnesota* R60, R345, R5, R7, and R595 (Ra, Rb_2, RcP^-, Rd_1P^-, and Re chemotypes, respectively) were purchased from List Biological Laboratories, Inc. (Campbell, Calif.). PCP-extracted rough LPS from *S. minnesota* R4 (Rd_2P^- chemotype) was generously provided by Penny Hitchcock (Rocky Mountain Laboratories, Hamilton, Mont.). Lipid A was prepared from *E. coli* O111:B4 LPS in my laboratory, as described previously (20), and the uniform triethylamine (TEA) salt form of lipid A (TEA-lipid A) from *S. minnesota* R595 LPS prepared by electrodialysis (7) was generously provided by Horst-Werner Wollenweber (Max Planck Institut, Freiburg, Federal Republic of Germany). Both of these lipid A preparations were found to contain less than 1% by weight of detectable 3-deoxy-D-mannooctulosonic acid. PCP-extracted *E. coli* J5 LPS (RcP^+ chemotype) was prepared in my laboratory. Alkaline-hydrolyzed LPS was prepared as described previously (21).

Buffers. A variety of buffers containing isotonic sodium barbital-buffered (pH 7.35) saline (VBS) were used for these studies: GVB^{++}, VBS with 0.1% gelatin–1.0 mM Mg^{2+}–0.15 mM Ca^{2+}; VBS-Mg^{2+}-Ca^{2+}, GVB^{++} without gelatin; VEM, VBS with 8 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid–2.0 mM Mg^{2+}–0.1% gelatin.

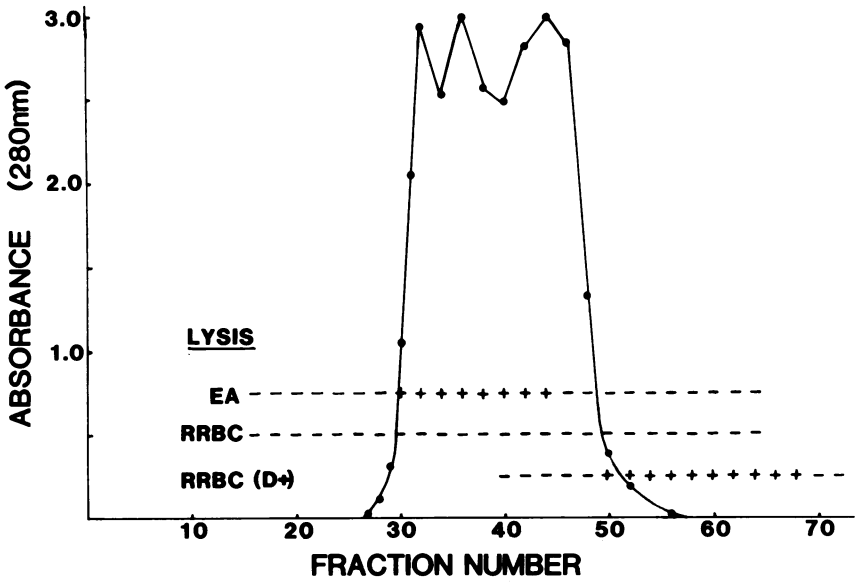

FIG. 1. Removal of factor D from NHS. NHS was chromatographed on Sephadex G75 as described in the text. Protein content of column fractions (10 ml) was monitored by measuring the absorbance at 280 mm (●). EA lysis was used to identify fractions containing hemolytic CPC. No lysis of RRBCs in VEM was detected for any fraction. D^+ fractions were detected by the lysis of RRBCs in VEM when the EA-lytic fraction pool was mixed with EA-nonlytic fractions, as described in the text.

Sera. The preparation of NHS was described previously (18). Sera were clarified by centrifugation at 25,000 × *g* for 1 h at 4°C to reduce the serum lipoprotein levels. This treatment of NHS had no significant effect on LPS-mediated complement activation or normal lysis of antibody-treated erythrocytes (EAs), but the decreased turbidity of the clarified sera increased the reproducibility between experiments by reducing background absorbance for the microtiter complement consumption assay described below. Where indicated, natural anti-RRBC antibodies were removed by absorption of 40 ml of serum with 4 ml of packed f-RRBCs (approximately 2×10^{11} f-RRBC total) for 1 h at −2°C. The absorption with f-RRBCs effectively removed the capacity of EDTA-treated serum to agglutinate RRBCs (9). In addition, unabsorbed D^- NHS serum was capable of antibody-dependent RRBC lysis in the absence of Ca^{2+} chelation, whereas the absorbed D^- NHS produced no such RRBC lysis. D^- NHS was prepared as described by Martin et al. (16), with slight modification. Briefly, 30 ml of clarified and, where indicated, f-RRBC-absorbed sera were applied to a Sephadex G75 (Pharmacia) column (9 by 18 cm) at 4°C and eluted with VBS-Mg^{2+}-Ca^{2+} buffer. The exclusion peak contained a fully functional CPC but contained no factor D, as determined by complete lysis of EA, and no detectable APC-dependent lysis of RRBCs in VEM (0.1 ml of column fraction plus 0.1 ml of 10^7 EAs or RRBCs per ml). Factor D-containing fractions (D^+) were detected by reconstitution of RRBC hemolytic activity when 50 μl of the exclusion volume was mixed with 50 μl of retained column fractions. The D^- NHS fractions and D^+ fractions were pooled, and each fraction pool was concentrated to the original serum volume. The approximate CPC 50% hemolytic complement units were ascertained for each batch of D^- NHS, and equivalent 50% hemolytic complement units of each batch of serum were used for all CPC assays. Typical recoveries for D^- NHS were >80% for C1 determined by radial immunodiffusion, as described by Ziccardi and Cooper (31); >39% of the 50% hemolytic complement activity; and >98% of C4, as determined by nephelometry. No significant differences in any of the results presented in this report were detected when serum from different donors was employed. The elution profile of a typical preparation is shown in Fig. 1. Fractions containing hemolytic activity are designated as EA lysis positive, and fractions containing factor D are designated as RRBC (D^+) positive.

Erythrocytes. Sheep erythrocytes (SRBCs) were purchased from either Colorado Serum Company (Denver, Colo.) or from Equitech (Atlanta, Ga.). Antibody-coated SRBCs were prepared by incubation of 10^9 SRBC per ml for 30 min at 37°C with a subagglutinating titer of rabbit anti-SRBC antiserum prepared in my laboratory. RRBCs were obtained from New Zealand White rabbits (Gillrey Rabbitry, Lawrenceville, Ga.) by bleeding from the central ear artery into a one-sixth volume of acid citrate-glucose anticoagulant. The fresh blood was then centrifuged for 5 min at 1,000 × *g*, and the supernatant and buffy coat were aspirated. The sedimented RRBCs were washed by centrifugation three times in VBS-EDTA, incubated for 1 h at 37°C in VBS-EDTA, and then washed an additional three times in VEM, as described by Platts-Mills and Ishizaka (23). The treated RRBCs were maintained at 4°C until use for experiments and were always used within 1 month after preparation. The RRBCs were routinely washed three times by centrifugation in VEM immediately prior to use. Formalin treatment of erythrocytes was performed by standard procedures (11). LPS-coated formalinized SRBCs (f-SRBCs) were prepared by incubation of 0.1 mg of alkaline-hydrolyzed LPS with 1 ml of packed f-SRBCs for 1 h at 37°C. The LPS-coated f-SRBCs were then washed by centrifugation three times in VEM buffer to remove unbound LPS.

Complement assay. The LPS dose-response assay described by Morrison and Kline (18) was adapted to a microtiter plate assay. Briefly, various LPS samples were mixed with an equal volume of double-strength GVB^{++}, the sample volume was adjusted to 120 μl with GVB^{++}, 100 μl of an appropriate dilution of D^- NHS was added to each sample, and samples were incubated at 37°C for 30 min. Then, 50 μl of 2×10^8 EAs were added to each sample, and

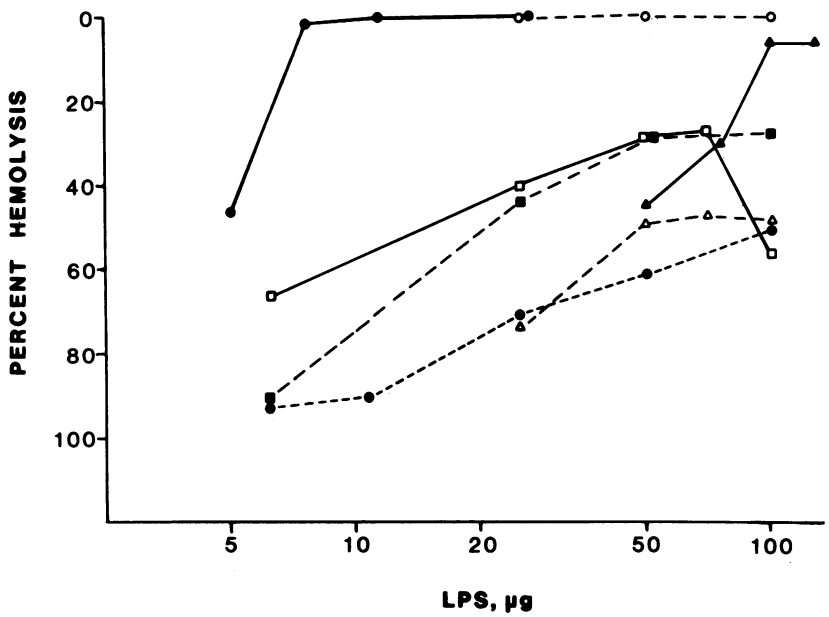

FIG. 2. Dose-response curves for the consumption of CPC activity in unabsorbed D^- NHS by R-chemotype LPS preparations. D^- NHS (100 μl) was mixed with LPS sample (120 μl) and incubated for 30 min at 37°C, and residual CPC activity was measured by the extent of EA lysis (50 μl of 2×10^8 EA/ml), as described in the text. The dose-response curves given are representative of three separate experiments. Symbols: —●—, R595 LPS; --○--, TEA-lipid A; --△--, R4 LPS; —▲—, R7 LPS; —□—, R5 LPS, --■--, J5 LPS; --●--, R345 LPS.

incubation was continued at 37°C for 30 min. Tubes were immediately chilled in an ice water bath, intact EAs were pelleted, and 200 μl of supernatants was transferred to 96-well, enzyme immunoassay flat-bottomed microtiter plates (Costar, Cambridge, Mass.). Absorbance was measured at 414 nm with a plate reader (Multiskan type 310C; Titertek; Flow Laboratories,McLean, Va.).

Immunofluorescence. NHS-treated f-RRBCs were washed five times with VBS, followed by treatment with a 1:10 dilution of either fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin G (IgG) [$F(ab')_2$-specific] antisera (Cappel Laboratories, Cochranville, Pa.) or FITC-conjugated goat anti-human IgM (μ chain-specific) antisera (Cappel). Nine volumes of fluorescent antibody reagent were added to one volume of packed f-RRBCs, and the mixture was incubated for 1 h at room temperature. Cells were then washed five times with VBS, and relative fluorescence intensities were determined with a fluorescence microscope (BH-RFL-W; Olympus Corp., New Hyde Park, N.Y.), a flow cytometer system (EPICs V; Coulter Electronics, Inc., Hialeah, Fla.) equipped with a multiparameter data acquisition and display system, or both. A laser (Spectro Physics 164; Spectro Physics, Mountain View, Calif.) was used as a light source, and total cell number was determined by filter-gated, forward-angle light scatter; and positive cells were detected with a green photomultiplier tube, with 515-nm blocking, 515-nm absorbance, and 530-mm short-pass filters in front of the light source.

RESULTS

It is well established that Re-chemotype LPS from *S. minnesota*, as well as lipid A isolated from a variety of LPS preparations, can activate the CPC by an antibody-independent mechanism and that O-antigen polysaccharide-rich smooth LPS preparations do not activate the CPC to any detectable extent. However, the minimal amount of polysaccharide contained in LPS micellar aggregates which is sufficient to block this lipid A-mediated CPC activation has not been fully elucidated. Previously, we found that some R-chemotype LPS structures activate the APC (S. W. Vukajlovich, J. Hoffman, and D. C. Morrison, submitted for publication); therefore, I felt it important to evaluate potential CPC activation by these LPS preparations in the complete absence of a functional APC system. For these experiments, D^- NHS was prepared by gel permeation chromatography, as described above. The interaction of a panel of *S. minnesota* rough mutant LPS chemotypes with D^- NHS provided the opportunity to examine in detail the relationship between LPS polysaccharide chain length and reduction in lipid A-mediated CPC activation.

The dose-response curves for CPC activation in D^- NHS by these LPS preparations is illustrated in Fig. 2, and several points are worthy of note. TEA-lipid A and a variety of R-chemotype LPS preparations were found to activate the CPC in D^- NHS. However, the LPS chemotypes which activated the CPC and contained greater amounts of polysaccharide than Re-chemotype LPS resulted in reduced levels of CPC consumption (data not shown), similar to that shown for R5 LPS (RcP^- chemotype) in Fig. 2. While this decrease in CPC consumption at high LPS doses was not investigated further, biphasic dose-response curves for CPC activation by LPS have been reported previously (22).

I considered the possibility that some of the activity observed in Fig. 2 might, therefore, be due to the presence of natural antibody to common antigenic determinants present on the LPS core oligosaccharide (1, 24, 32). Advantage was taken of earlier observations indicating that both R-LPS chemotypes (17) and RRBCs (26) share carbohydrate determinants which can be recognized by the lectin concanavalin A. This suggests that natural anti-RRBC antibodies in NHS (9, 23) could be cross-reactive with core oligosaccharide determinants and absorption of serum with f-RRBC could

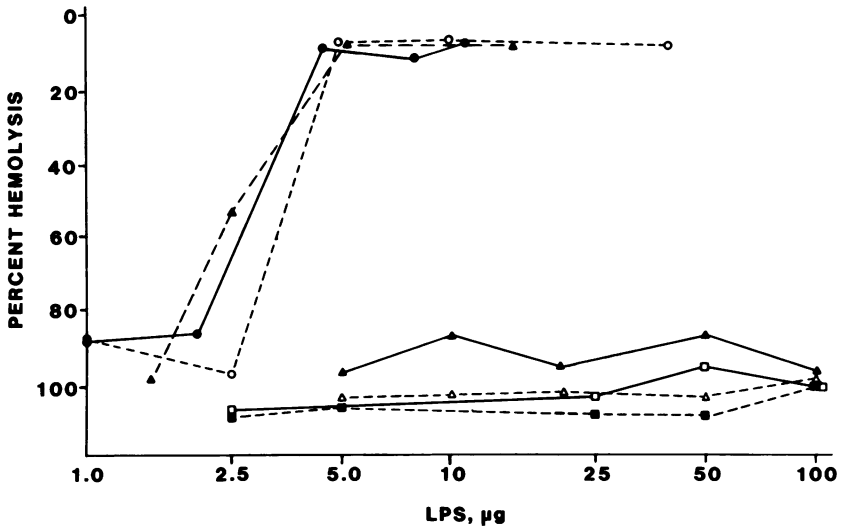

FIG. 3. Consumption of CPC activity in absorbed D⁻ NHS by R-chemotype LPS preparations. CPC consumption was measured as described in the legend to Fig. 2, except that serum was absorbed with f-RRBCs, as described in the text. Dose-response curves given are representative of three separate experiments. Symbols: --○--, TEA-lipid A; --▲--, lipid A plus TEA; —●—, R595 LPS; —▲—, R7 LPS; --△--, R4 LPS; —□—, R5 LPS; --■--, R345 LPS.

potentially remove natural anti-LPS antibodies present in NHS. For these experiments, D⁻ NHS was extensively absorbed with f-RRBC at −2°C, as described above, and the LPS preparations shown in Fig. 2 were reevaluated for CPC activation.

LPS-mediated CPC activation of pretreated D⁻ NHS by the *S. minnesota* chemotypes (Fig. 3) produced a strikingly different pattern of CPC consumption compared with the results obtained with unabsorbed D⁻ NHS. Following absorption, only lipid A and R595 LPS were capable of activating the CPC, suggesting that the prior absorption of serum removed factors which bind R-LPS chemotypes and generate a CPC-activating complex. I did not observe any difference in the capacity of absorbed sera to lyse antibody-sensitized SRBCs in comparison with control sera, which would also suggest that the absorption procedure does not markedly deplete CPC-specific components. In addition to the LPS preparations shown in Fig. 3, a spectrum of additional polysaccharide-containing LPS preparations was tested, and it also failed to activate the CPC with absorbed serum. The LPS preparations included LPS from *E. coli* J5 and O55 strains, as well as all of the O55 LPS fractions, F1 through F5, described elsewhere (28), LPS from *Serratia marcescens*, and LPS from *Y. enterocolitica*. Thus, I conclude that the presence of a single heptose sugar in Rd_2-chemotype LPS, as compared with the Re-chemotype LPS, is sufficient to totally abrogate the capacity of LPS preparations to initiate CPC activation by lipid A.

To characterize, in part, the specific component removed by absorbing NHS with f-RRBC, NHS-treated f-RRBCs were subsequently analyzed for their capacity to bind FITC-conjugated goat anti-human immunoglobulin (Fig. 4). Both FITC-anti-human IgM and FITC-anti-human IgG were bound by NHS-treated f-RRBCs (Fig. 4a and b, respectively), indicating that both IgM and IgG antibodies are removed from NHS by f-RRBC absorption. Neither formalinized human erythrocytes nor f-SRBC absorbed any detectable IgM or IgG antibodies in NHS (data not shown). In addition, the data presented in Fig. 4a and b indicate that the majority of anti-RRBC antibodies are removed from NHS by preabsorption with f-RRBCs. f-SRBCs coated with alkaline-hydrolyzed Rd_1-chemotype LPS also bound both IgG (Fig. 4c) and IgM antibodies (data not shown), and preabsorption with f-RRBCs removed a significant amount of the antibody that was reactive with Rd_1-chemotype LPS. Similar results were obtained with *E. coli* J5 LPS (Rc chemotype)-coated f-SRBCs (data not shown). While some antibody directed against Rd_1-chemotype LPS-coated f-SRBCs could still be detected following absorption of NHS with f-RRBCs, the concentrations of these antibodies were sufficiently reduced such that no antibody-dependent CPC activation by R-chemotype LPS could be detected (Fig. 3).

DISCUSSION

The results of the experiments presented here demonstrate that only isolated lipid A and Re-chemotype LPS activate the CPC by a lipid A-dependent, antibody-independent mechanism. The additonal monosaccharide L-glycero-D-mannoheptose present in Rd_2-chemotype LPS, as compared with the Re-chemotype LPS structure, appears to be sufficient to block the appropriate CPC-activating determinant(s) located in the lipid A portion of the molecule. While polyanionic surfaces are known to activate the CPC (14), the precise nature of the lipid A and Re-chemotype LPS determinants involved in C1 activation has yet to be identified. However, the requirement for the TEA salt form of lipid A for the activation of CPC indicates that the tertiary arrangement of subunits within lipid A aggregates is, in part, a significant parameter.

The experiments reported here also provide evidence that natural anti-RRBC antibodies found in all NHS (9, 23) cross-react with antigenic determinants on LPS polysaccharides. Data from this study demonstrate that RRBC membranes express surface antigens which are sufficiently cross-reactive with LPS core polysaccharide determinants to effectively absorb these LPS-binding molecules from serum. Whether or not additional nonimmunoglobulin molecules which cross-react with both RRBCs and LPS are removed from NHS by absorption with RRBCs remains to be determined. It is noteworthy in this respect that earlier studies have shown that concanavalin A will agglutinate

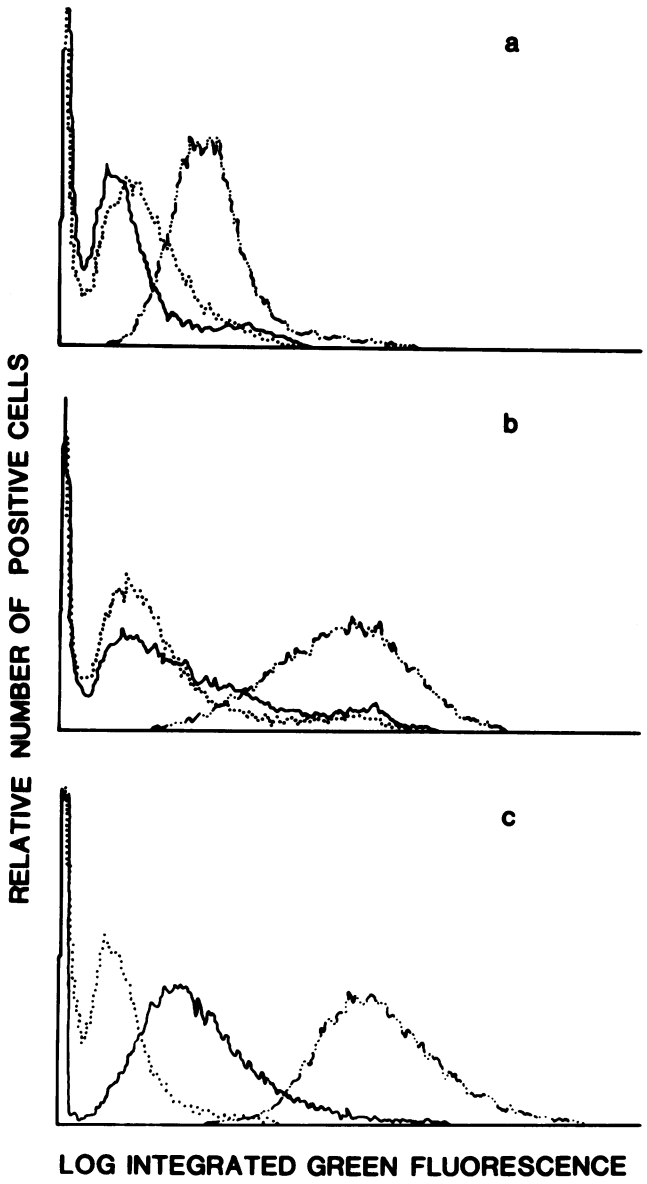

FIG. 4. Detection of natural anti-RRBC antibodies in NHS by flow cytometry. Serum-treated f-RRBCs were incubated with either FITC-conjugated goat anti-human IgG or IgM, as described in the text. (a) Symbols: · · · ·, FITC-conjugated goat anti-human IgG bound to f-RRBC not treated with serum, – · · · –, f-RRBC treated with NHS; ——, f-RRBC treated with NHS preabsorbed with f-RRBC. (b) Same as described above for panel a, except that FITC-conjugated goat anti-human IgM was used. (c) Symbols: · · · ·, FITC-conjugated goat anti-human IgG bound at alkaline hydrolyzed Rd_1-chemotype LPS-coated f-SRBC (Rd-SRBC) not treated with NHS; – · · · –, Rd-SRBC treated with NHS; ——, Rd-SRBC treated with Rd-SRBC-absorbed NHS.

RRBCs (26) and also will bind to LPS core polysaccharide determinants (17). In addition, Galili et al. (9) have described a natural IgG antibody in human sera with anti-α-galactosyl specificity which was shown to bind to α-galactosyl residues on RRBCs. Although not tested, such an antibody should also bind Rb_2 LPS which contains two terminal α-galactose residues. Thus, it is likely that natural antibodies against terminal galactose, glucose, or glucosamine residues in NHS may bind many R-chemotype LPS preparations and yield immune complexes that are capable of activating the CPC. The experiments presented here do not exclude the possibility that nonimmunoglobulin, noncomplement proteins bind LPS and either contribute to or interfere with the ability of LPS to activate complement (1, 13). Several serum proteins which bind LPS have been described. While effects of LPS binding by serum albumin (30) or high-density lipoproteins (27) with respect to complement activation are not clear, other serum molecules which bind LPS to form CPC-activating complexes have been described. Kawakami and co-workers (12, 13) have recently described a highly conserved bactericidal factor present in the sera of all vertebrates tested. This factor binds specifically to Ra-chemotype LPS from several strains of enterobacteria to form CPC-activating complexes (12). Brade and Brade (1) have described a serum factor which binds a determinant of the inner core region to LPS to form CPC-activating complexes. This factor binds to the 3-deoxy-D-mannooctulosonic acid region present in all LPSs tested except for Re-chemotype LPS (1). NHS is, therefore, rich in both natural antibodies and factors which interact with LPSs and activate the CPC by binding to LPS determinants which normally block lipid A-mediated activation of the CPC by purified LPS. It is important to note that if the factors characterized by Kawakami and co-workers (12, 13) and Brade and Brade (1) do not bind to f-RRBCs, they would be removed in the preparation of D^- NHS because of their low molecular weight.

Of critical importance to the conclusions of this report is the fact that, in the absence of antibody, other LPS-binding factors, or both, the Re-chemotype LPS is a potent activator of the CPC, whereas the Rd_2-chemotype LPS is without detectable activity. However, recent studies have shown that C1q and C1 bind to all of the R-chemotype LPS structures (3, 25). While these results appear to conflict with the data presented here, the ability to activate C1 does not correlate with either C1 binding or C1-binding affinity (4). This suggests that C1 binds to non-Re, R-chemotype LPS preparations but is not activated. The ability of L-glycero-D-mannoheptose monosaccharide to abrogate CPC activation by Rd_2-chemotype LPS was somewhat surprising, because earlier investigations by Galanos and Luderitz (7) have demonstrated that the Re- and Rd-chemotype LPSs have similar physical-chemical properties which were also reflected by their capacity to mediate complement activation. In these studies, the Ca^{2+} and Mg^{2+} present in guinea pig serum was thought to induce the TEA forms of both the Re and Rd-chemotype LPSs to reaggregate to relatively high-molecular-weight forms which could activate serum complement, whereas the remaining R-chemotype LPS and smooth LPS preparations remained in their low-molecular-weight inactive forms. However, recent experiments by Brandenburg and Seydel (2) have demonstrated significant differences between the phase behavior of lipid A, Re-chemotype LPS, and Rd_2-chemotype LPS. We have found that Rd_2- and Rd_1-chemotype LPS activate the alternative pathway of complement (Vukajlovich et al., submitted for publication). Therefore, excluding possible differences in guinea pig and human C1 activation, it is probable that the anticomplementary activity of Rd-chemotype LPS observed by Galanos and Luderitz (7) resulted from APC activation, serum factor(s)-dependent CPC activation, or both.

I conclude from the results of the experiments reported here that activation of the CPC by LPS is restricted to the Re chemotype and isolated lipid A. It is important to emphasize, however, that this conclusion pertains only to lipid A-

dependent activation of the CPC. As a consequence, I do not preclude the possibility that an oligosaccharide structure in the O antigen of a specific LPS preparation may also interact with C1 by an antibody- and lipid A-independent mechanism. There is, in fact, good evidence that certain polysaccharides can bind and activate C1 (14). Such a mechanism may, at least in part, contribute to earlier observations (15, 18) which suggest that polysaccharide-containing LPS preparations can activate the CPC. Nevertheless, data from this study do not support the concept that polysaccharide-containing LPSs activate the CPC by interacting with lipid A.

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