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Selective Pressures and Lipopolysaccharide Subunits as Determinants of Resistance of Clinical Isolates of Gram-Negative Bacilli to Human Serum

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Differences in molecular composition of lipopolysaccharides (LPS) between serum-sensitive (S) clinical isolates of *Escherichia coli* and serum-resistant (R) clones derived by serial passage in serum were demonstrated to determine sensitivity or resistance to killing by normal human serum (NHS). LPS from R clones had a greater proportion of higher-molecular-weight, more highly O-antigen-substituted subunits than LPS from their serum S parents. Utilization of a liposomal model with inserted LPS simulating bacterial cell walls established LPS as the site of serum bactericidal action. Liposomes containing S LPS were lysed, while liposomes containing R LPS were unaffected by NHS. R and S LPS were fractionated into higher (F1)- and lower (F2)-molecular-weight fractions. Liposomes containing R LPS or the F1 fraction of S and R LPS were not lysed by serum. Liposomes containing the F2 fraction of S or R LPS were lysed by serum analogous to that observed with liposomes containing intact S LPS. These findings establish LPS to be one site of serum bactericidal activity and demonstrate that the higher-molecular-weight, highly O-antigen-substituted LPS subunits mediate resistance to killing by NHS.

Gram-negative bacilli (GNB) have become the most frequent cause of nosocomial infections and death from infections in this country during the past 4 decades (11). Complex interrelations between host defense mechanisms, therapeutic measures, and bacterial factors influence the development and outcome of such infections (1, 11). Although adherence mechanisms and exotoxin production appear crucial for colonization and infection at some body sites (2, 7, 24), other possible virulence factors of GNB are less well defined. Resistance to rapid killing by normal human serum (NHS) appears important for development of systemic GNB infections since most isolates from the gastrointestinal tract are sensitive (S), while isolates from systemic clinical infections are almost uniformly resistant (R), ≥ 85%, to killing by serum (12, 19).

Mechanisms responsible for the almost uniform resistance of GNB isolated from clinical infections and the exact mechanism(s) responsible for killing by NHS have not been delineated, although several explanations have been proposed. Cell wall components, particularly lipopolysaccharide (LPS), K or capsular antigens, and outer membrane proteins have been postulated to be sites sensitive to and responsible for resistance to killing by NHS (14, 16, 17, 22, 26, 28). Serum R GNB have been shown to contain more longer-chain, highly O-antigen-substituted LPS subunits than S strains (4, 5, 22, 25, 26, 28), but laboratory-maintained or -derived rough mutants rather than fresh clinical isolates have often been used to represent serum S strains. Goldman et al. (5) also demonstrated that serum R clones could be selected from laboratory-maintained S strains by serial passage in serum. Others have suggested that plasmid-mediated alterations in outer membrane proteins are associated with slight increases in serum resistance (14, 16). Capsular antigen has also been proposed to mediate resistance (17). Classically, killing of intact bacteria has been the standard assay of the activity of NHS, but the multiple cellular components present hamper exact identification of the precise site of killing.

These studies examine the selection of R clones by serial passage of fresh clinical isolates of S GNB in NHS and utilize the liposomal model of Kinsky et al. (9) to clearly establish LPS as a site of serum bactericidal activity. Use of this multilamellar liposomal model allowed identification of LPS subunits responsible for conferring resistance to killing. This model provides an ideal system for examination of the role of various bacterial and serum components involved in serum bactericidal activity.

MATERIALS AND METHODS

Bacterial strains. Clinical isolates of serum S *Escherichia coli* (strain, serotype: 39, O4:K-12; 109, O6; 227, O18ac:K1; 248, O6:K2; 337, O75; and 348, O7) were obtained from the Microbiology Laboratory of University Hospital, Boston, Mass. The strains were identified as serum S and were serotyped as described previously (12).

NHS pool. Blood was obtained by venipuncture (50 ml each) from 30 normal volunteers, allowed to clot, and separated in the cold, sera were pooled, and 1.0-ml aliquots were frozen at −60°C. This pool was used in the selection of serum R clones and as a source of complement and antibody in serum bactericidal assays.

Selection of R clones. Each serum S *E. coli* was inoculated in 2 ml of brain heart infusion (BHI) broth and incubated overnight at 35°C. Samples (0.001 ml) were inoculated into fresh BHI broth containing 50% NHS and incubated at 35°C

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for 2 h. These subcultures were screened for the presence of serum R clones by the agar plate method of Fierer et al. (3). Bacteria (serum R) growing within the area where NHS had been deposited were inoculated into BHI broth and incubated overnight. The same sequence of incubation in 50% NHS, subculturing, and selection by the agar plate method was repeated until there was a solid bacterial lawn in the area where NHS had been added. Once a serum R population was obtained, cultures were serially passed 15 to 20 times in serum-free broth to ensure their stability.

Bactericidal assay. Bacterial killing was quantitated by a standard microtiter plate assay technique (12). Bacteria grown in BHI broth to the log phase (4 h) were diluted logarithmically in phosphate-buffered saline containing 5×10^{-4} M $MgCl_2$ and 10^{-3} M $CaCl_2$ (PBS^{++}). Duplicate 1.0-ml samples of 10^{-6} dilutions were plated for counts of the original inoculum. Microtiter plates (Linbro; Flow Laboratories, Inc., McLean, Va.) were inoculated with 0.05-ml samples of bacteria (approximately 250), 0.05 ml of PBS^{++}, and 0.1 ml of NHS or decomplemented NHS (56°C for 30 min) and incubated for 2 h. Duplicate 0.1-ml samples were plated overnight for bacterial counts. The percentage of the original inoculum surviving after 2 h of incubation was calculated.

LPS. Each bacterial strain (serum S parent and serum R clone) was grown in 15 liters of Casamino Acids broth (Difco Laboratories, Detroit, Mich.) supplemented with yeast extract and glucose (Sigma Chemical Co., St. Louis, Mo.). After 16 to 18 h of incubation, bacteria were killed and precipitated by the addition of 2.5 volumes of acetone. Precipitated bacteria were washed three times in acetone and air dried, and LPS was extracted by the hot phenol-water method of Westphal et al. (29), dialyzed, and lyophilized.

Radiolabeled LPS. Bacteria were grown with the addition of ^{3}H-labeled sodium acetate (5 mCi/liter; New England Nuclear Corp., Boston, Mass.), and LPS was extracted as described above (29).

Saponification of LPS. LPS used for incorporation into liposomes was saponified by treatment with 0.1 N NaOH (2 mg/ml) at 35°C for 18 h. The pH was adjusted to 7.0 to 7.2 with 1 N HCl, and the saponified LPS was dialyzed against distilled water for 3 days and lyophilized (13).

PAGE. Polyacrylamide gel electrophoresis (PAGE) of LPS was performed in 0.75-mm-thick 14% T gels with Tris glycine buffer (pH 8.4) containing 0.1% sodium dodecyl sulfate by the method of Laemmli (10). Samples dissolved in a digestion buffer of 0.18 M Tris (pH 6.8), 90 mM dithiothreitol, sodium dodecyl sulfate, and 25% urea were heated to 100°C for 10 min or unheated, and 0.01 ml of 1-mg/ml concentration was applied to gels. Gels were run at a constant current of 15 mA in an LKB model 2001 vertical electrophoresis unit, stained with silver reagent, and dried. All chemicals and reagents were of electrophoresis grade (Bio-Rad Laboratories, Richmond, Calif.).

Sephadex G-200 chromatography of LPS. LPS was fractionated on a Sephadex G-200 column by a modification of the method of Vukajlovich and Morrison (27). LPS from both serum S and R bacteria was disaggregated in 0.1 M Tris buffer (pH 8.5) containing 0.6% sodium deoxycholate (DOC) at 50 mg/ml. A 2-ml sample was applied to a Sephadex G-200 column (2.6 by 55 cm; Pharmacia, Inc., Piscataway, N.J.), equilibrated with the same buffer, and eluted at 35°C at a rate of 12 ml/h. Fractions of 2.5 ml were collected and analyzed for hexose and 3-deoxy-D-*manno*-octulosonic acid (KDO) content.

Chemical methods. Hexose was determined by either the anthrone method (23) or the cysteine-sulfuric acid method (30) with glucose as the standard. KDO was determined by the thiobarbituric acid method (18) with 2-keto-3-deoxyoctonate (Sigma) as a standard.

Liposome preparation. Stock solutions of veronal-buffered saline (VBS), $MgCl_2$-$CaCl_2$, gelatin-VBS (GVBS), and GVBS containing 0.125 ml of $MgCl_2$-$CaCl_2$ ($GVBS^{++}$) were prepared. Three lipids (Sigma), dimyristyl phosphatidylcholine (25 mM in chloroform), cholesterol (75 mM in chloroform), and dicetylphosphate (3 mM in 1:1 [vol/vol] methanol-chloroform), were mixed in a 10-ml pear-shaped boiling flask in a molar ratio of 2:1.5:0.22 as described by Kinsky et al. (9) and Kataoka et al. (8). Chloroform and methanol were removed by evaporation on a rotary evaporator (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N.J.), producing a thin film of dried lipids on the lower portion of the flask which was placed in a desiccator jar under vacuum for 1 h to complete drying. Appropriate amounts of LPS and 15 μCi of ^{51}Cr (as sodium chromate; New England Nuclear Corp.) in a total volume of 0.2 ml of GVBS were added, and the flask was agitated vigorously (Vortex Genie mixer; The Vortex Manufacturing Co., Cleveland, Ohio) for 5 min. The multilamellar liposomes formed were passed through a column of Sephadex G-25 (1.6 by 30 cm; Pharmacia Fine Chemicals, Piscataway, N.J.) in $GVBS^{++}$ to separate ^{51}Cr-containing liposomes from free ^{51}Cr. Fractions (1 ml) were collected and counted in a gamma counter (model 1197; Searle Analytic, Des Plaines, Ill.), and the entire ^{51}Cr-labeled liposome fraction was pooled, washed in GVBS, and suspended in 1 ml of $GVBS^{++}$. The liposome suspension (200 μl) was incubated at 35°C for 30 min with one of the following: 20% NHS, 20% decomplemented NHS, or $GVBS^{++}$ alone in a volume of 1 ml. Each mixture was chromatographed on Sephadex G-25, and 1-ml fractions were counted in a gamma counter to assay

TABLE 1. Bactericidal activity of NHS against six strains of serum or resistant clinical isolates of *E. coli* and derived or resistant serum clones

Strain (serotype)	Initial bacterial count/ml	Count/ml after serum incubation	% Killed
39 (O4:K-12)			
Parent strain (serum S)	920	72	92
Clonal derivative (serum R)	960	810	16
109 (O6)			
Parent strain (serum S)	1,210	120	91
Clonal derivative (serum R)	1,080	920	15
227 (O18ac:K1)			
Parent strain (serum S)	660	60	91
Clonal derivative (serum R)	840	3,600	0
248 (O6:K2)			
Parent strain (serum S)	1,110	110	90
Clonal derivative (serum R)	1,260	1,060	16
337 (O75)			
Parent strain (serum S)	980	240	76
Clonal derivative (serum R)	1,040	2,510	0
348 (O7)			
Parent strain (serum S)	780	56	93
Clonal derivative (serum R)	810	660	19

liposome-bound ^{51}Cr and free ^{51}Cr released from the liposomes.

RESULTS

Selection of serum R clones. Serum R clones were selected from each of six serum strains of *E. coli* of various O-antigen serotypes (strain, serotype: 39, O4:K-12; 109, O6; 227, O18ac:K1; 248, O6:K2; 337, O75; and 348, O7) by multiple passages in serum-containing BHI broth and agar plate screening. The R clones were serially passed in serum-free medium to ensure the stability of resistance, and identity of the parent S strains and R clones was confirmed by biotype and antibiotic susceptibility patterns and serotyping (Centers for Disease Control, Atlanta, Ga.). Table 1 illustrates the magnitude of bacterial killing of the parent serum S and serum R clone by NHS. NHS produced 90% or greater killing with five of the six parent strains, while 75% killing occurred with the sixth (337) strain. In contrast, less than 20% of the inoculum of four R clones (39, 109, 248, and 348) was killed by NHS, while the remaining two R clones (227 and 337) multiplied. Two pairs of strains (109 and 227) were selected for further evaluation of the basis of resistance to NHS.

PAGE. PAGE patterns of LPS from the two serum S parent strains and their R clones are illustrated in Fig. 1. A typical ladder formation of subunit structure of LPS from smooth GNB is seen, with densely staining broad bands at the bottom of the gel, representing core components, accounting for at least 50% of the LPS. The number and density of bands in samples heated to 100°C for 10 min (lanes 2, 4, 6, and 8) did not differ from that of unheated samples (lanes 1, 3, 5, and 7). The major differences occur in the banding pattern at the top of the gel (longer-chain, highly O-antigen-substituted, higher-molecular-weight components), where LPS from R GNB exhibited more densely staining bands. Two dense bands (c) at the top of the gel of LPS from R 109 are barely visible in LPS from S 109 (lanes 1 and 2). Scanning densitometry, performed through the courtesy of Fred Wingerath, LKB Instruments, Inc. (Rockville, Md.), confirmed the greater intensity of bands in the higher-molecular-weight region of LPS from R clones 109 and 227 than in LPS from S parent strains. Table 2 lists the cumulative density of three distinct clusters of bands, labeled a, b, and c on the PAGE gel in Fig. 1, in these areas, confirming the greater content of more highly O-antigen-substituted, high-molecular-weight subunits in LPS from R clones than in LPS from their S parents. The c area (highest molecular weight) density of R 109 exceeds that of S 109 by approximately 20-fold.

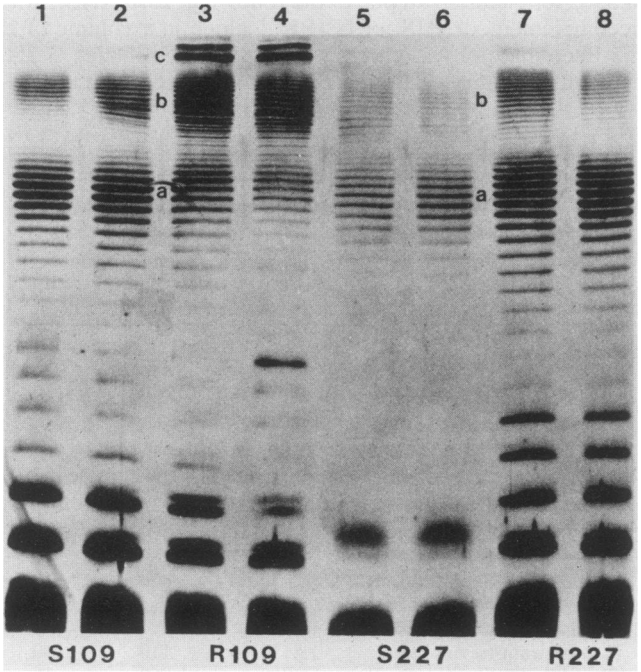


FIG. 1. PAGE of untreated LPS (lanes 1, 3, 5, and 7) and samples heated to 100°C for 10 min (lanes 2, 4, 6, and 8) of two serum S clinical isolates and R clones derived from them. Major differences are seen in the upper portion of the gels, areas a, b, and c from *E. coli* 109 and a and b from *E. coli* 227, representing the higher-molecular-weight subunits of LPS. Much denser staining is seen in region a and b of the R clones than with serum S 109 and 227, while area c, the heaviest band of R 109, is barely seen in LPS from its parent S strain.

TABLE 2. Comparison of intensity of bands in the higher-molecular-weight regions of the PAGE gel

LPS	Region a[a]	Region b[a]	Region c[a]
109			
S	580 (60)	390 (40)	2.2 (0.2)
R	468 (39)	584 (49)	40 (12)
227			
S	273 (66)	143 (34)	
R	790 (72)	315 (28)	

[a] Numbers represent absolute density readings.
[b] Numbers in parentheses represent percentage of total intensity of all the bands in the higher-molecular-weight regions.

Lysis of liposomes containing LPS. (i) Liposome model. The

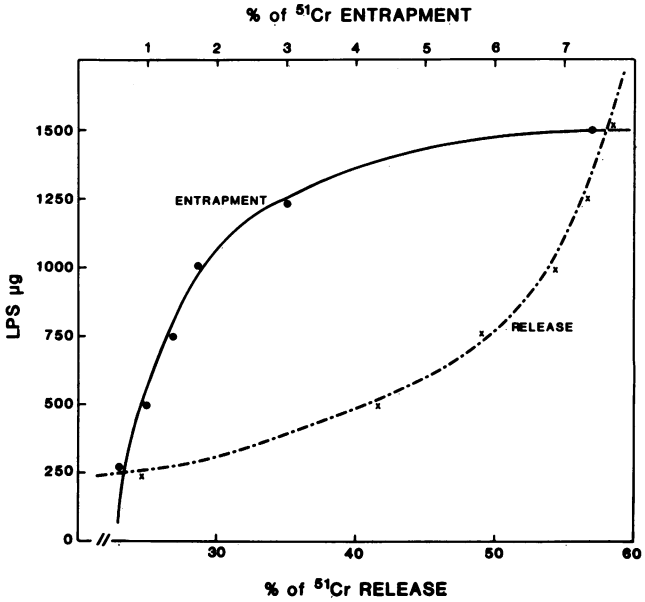


FIG. 2. Dose-response curve of entrapment and release of ^{51}Cr from liposomes prepared with various amounts of LPS after treatment with NHS. Percent entrapment and release of ^{51}Cr increase with increasing doses of LPS with both approaching maximum at concentrations slightly greater than 1 mg.

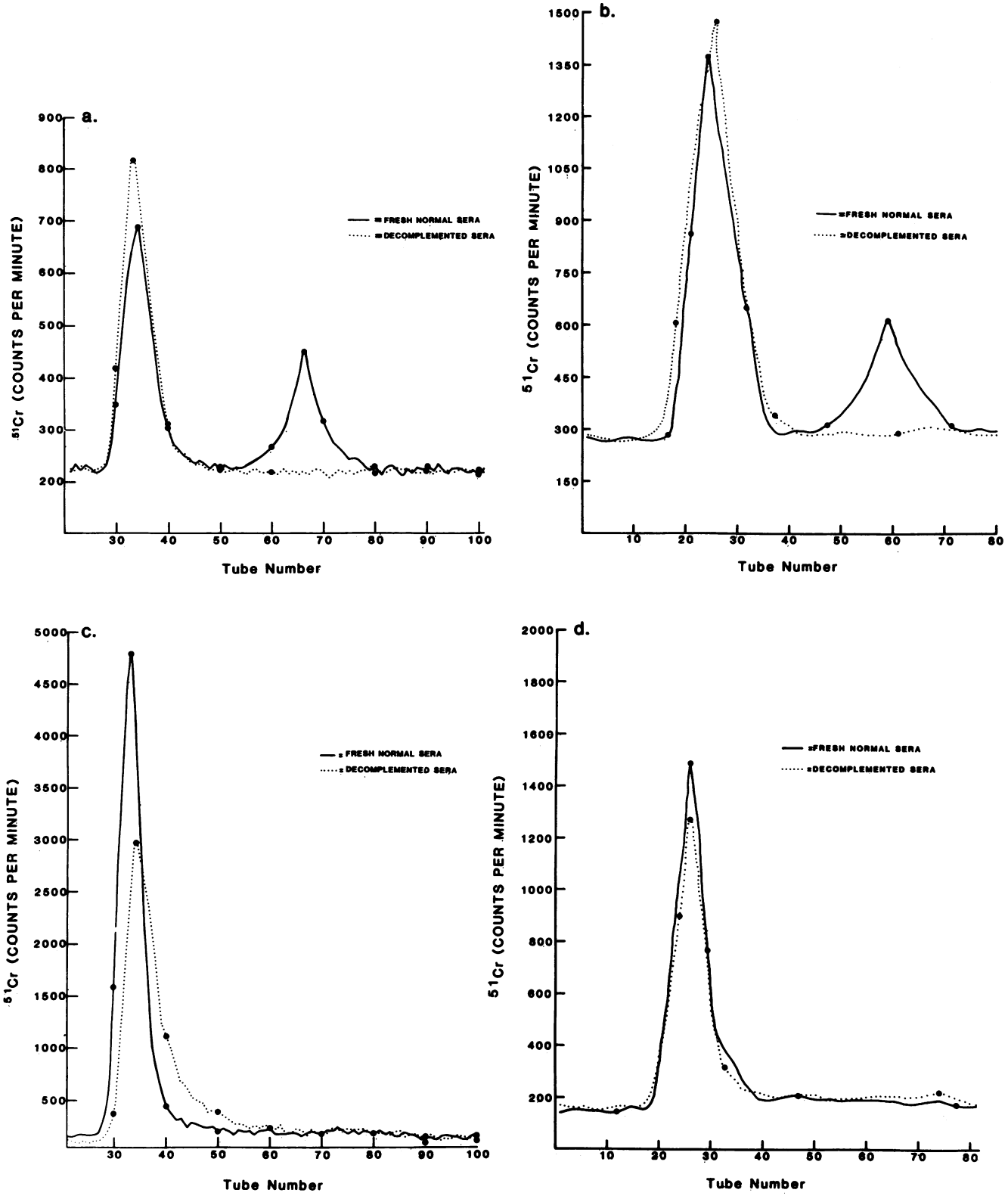


FIG. 3. Distribution of ^{51}Cr after incubation of liposomes containing ^{51}Cr and LPS (a, S 109; b, S 227; c, R 109; d, R 227) with serum (solid line, NHS; broken line, heat-inactivated serum). Only a single peak, representing unlysed liposomes, is seen with heat-inactivated serum (a–d) with each LPS and with liposomes containing LPS from R 109 (c) and R 227 (d) incubated with NHS. Two peaks are seen with liposomes containing LPS from S 109 (a) and S 227 (b) after incubation with NHS, with the second peak representing free ^{51}Cr released from lysed liposomes.

dose response of entrapment and release (Fig. 2) was determined by measuring ^{51}Cr release after incubation of liposomes with variable amounts of S 227 LPS. Since both entrapment and release approached maximum at an LPS concentration of 1 mg, this dose was chosen for incorporation for further studies. The applicability of these findings to LPS from R GNB was examined with ^{3}H-radiolabeled LPS from S and R 227. Liposomes were prepared with 1-mg amounts of each [^{3}H]LPS and separated from unincorporated LPS by passage through a Sephacryl 1000 column (1.6 by 40 cm; Pharmacia Fine Chemicals). Radioactivity, measured in a liquid scintillation counter (Tracor Analytic Delta 300) with Aquasol as the scintillator, demonstrated that incorporation of [^{3}H]LPS from both S and R 227 was virtually identical (12%).

(ii) Lytic assays. The differences in subunit composition of LPS from serum S and serum R clones suggested, but did not establish, LPS as the site of killing by NHS. The actual role of LPS as the site of the bactericidal activity was established by using liposomes into which LPS from serum R and S GNB was incorporated along with ^{51}Cr as a release marker for lysis. Liposomes were prepared from LPS from two serum S parent strains (S 109 and S 227) and two serum R clones (R 109 and R 227) and separated from free ^{51}Cr by passage through a Sephadex G-25 column (1.6 by 30 cm). Separation curves demonstrated two peaks, with the first peak containing radiolabeled liposomes and the second peak containing free ^{51}Cr. The liposome preparations were washed with GVBS to remove excess LPS and suspended in $GVBS^{++}$, and 0.2-ml amounts were incubated in NHS, decomplemented NHS, or $GVBS^{++}$ for 30 min at 35°C. Liposomes without LPS were not damaged and ^{51}Cr was not released after incubation with NHS. Fig. 3a, b, c, and d demonstrate results observed with the four LPS preparations tested. ($GVBS^{++}$ controls did not differ from results with decomplemented serum and are not shown.) Fig. 3a and b show release of ^{51}Cr from liposomes containing LPS from the two serum S parent strains, with the initial peak representing ^{51}Cr remaining in unlysed liposomes, while the second peak demonstrates release of approximately 50% of incorporated ^{51}Cr from lysed liposomes. In contrast, liposomes containing LPS from serum R clones (Fig. 3c and d) were not lysed by NHS, and no secondary peaks of ^{51}Cr are seen. In all cases, incubation with decomplemented NHS did not result in lysis of the liposomes and release of free ^{51}Cr.

Sephadex G-200 column chromatography of LPS. Sephadex G-200 chromatography of DOC-disaggregated LPS from S and R GNB was performed, and each column fraction was assayed for hexose (anthrone method) (Fig. 4, heavy solid lines) and KDO (Fig. 4, vertical lines), which was determined qualitatively (− to +++++) since DOC precipitated during the reaction complicated quantitative determination (Fig. 4). Hexose content of the higher-molecular-weight F1 fractions exceeded that of the F2 fractions from both strains, and the hexose content of the F1 fraction of LPS from R clones greatly exceeded that of the F1 fraction from S parent strains. The lower-molecular-weight fractions of LPS from both S and R GNB contain more KDO than the higher-molecular-weight fractions. The eluted fractions were pooled into two large fractions, F1 (higher molecular weight) and F2, extensively dialyzed to remove DOC, and lyophilized. Table 3 lists the amounts recovered, the absolute quantities of hexose and KDO, and hexose/KDO ratios of all four LPS preparations and their fractions. LPS from R strains contained approximately 2.5 times the amount of the F1 fraction but less than 0.5 the quantity of the F2 fraction as LPS from S strains. Hexose/KDO ratios did not differ materially between whole LPS of all strains or between the F1 or F2 fractions from serum S or R LPS, respectively. However, hexose/KDO ratios of F1 fractions, approximately 20:1, were four to six times greater than those of F2 fractions, approximately 4:1. PAGE of each of these fractions demonstrated that the F1 and F2 fractions corresponded to the higher- and lower-molecular weight regions (Fig. 5) previously described in Fig. 1.

Lysis of liposomes containing LPS fractions. These results led to direct examination of the role of the higher- and lower-molecular-weight LPS fractions in determining susceptibility to lysis by NHS. Fractions F1 and F2 were obtained from LPS from S and R strains, and 1 mg of each was incorporated into liposomes, as with the parent LPS, and treated with NHS. Similar concentrations of the F1 fractions from both S and R *E. coli* 109 protected liposomes from lysis by NHS (Fig. 6a and b), with no release of ^{51}Cr from liposomes containing fraction F1 from LPS of either serum S or R strains. In contrast, liposomes containing fraction F2 from serum S and R strain 109 were lysed, and ^{51}Cr was released (Fig. 6c and d). Similar results were obtained with F1 and F2 fractions from LPS of serum S strain 227 and its R clone. Liposomes containing F1 fractions from both LPS preparations were not lysed, while those containing the F2 fractions from both strains were lysed. The amount of ^{51}Cr released from liposomes containing F2 fractions were ±60% which was slightly greater than that of unfractionated LPS from serum S strains. Taken in toto, these observations provide convincing evidence that serum resistance in these isolates is mediated by the highly O-antigen-substituted, higher-molecular-weight subunits of LPS and that these are present in greater amounts in serum R GNB than in serum S GNB.

DISCUSSION

Since the description of Neisser and Wechsberg (15) of the bactericidal activity of NHS, almost all gram-negative bacterial species have been shown to be killed by sera from many animal species (26). Both the complement system and antibody appear necessary for killing of smooth strains of

TABLE 3. Hexose and KDO analysis of LPSs and their DOC column fractions

Sample	Dried wt (mg)	Hexose[a] (μg/mg)	KDO[b] (μg/mg)	Ratio Hexose/KDO
S 109		160	16	10.1
F1	14	230	11	21:1
F2	36	170	36	5:1
R 109		180	23	8:1
F1	32	278	14	20:1
F2	17	115	29	4:1
S 227		123	24	5:1
F1	13	190	10	18:1
F2	37	70	21	3:1
R227		170	23	7:1
F1	34	210	10	20:1
F2	16	125	39	3:1

[a] Cysteine-sulfuric acid method of Wright and Rebers (30).
[b] Thiobarbituric acid method of Osborn (18).

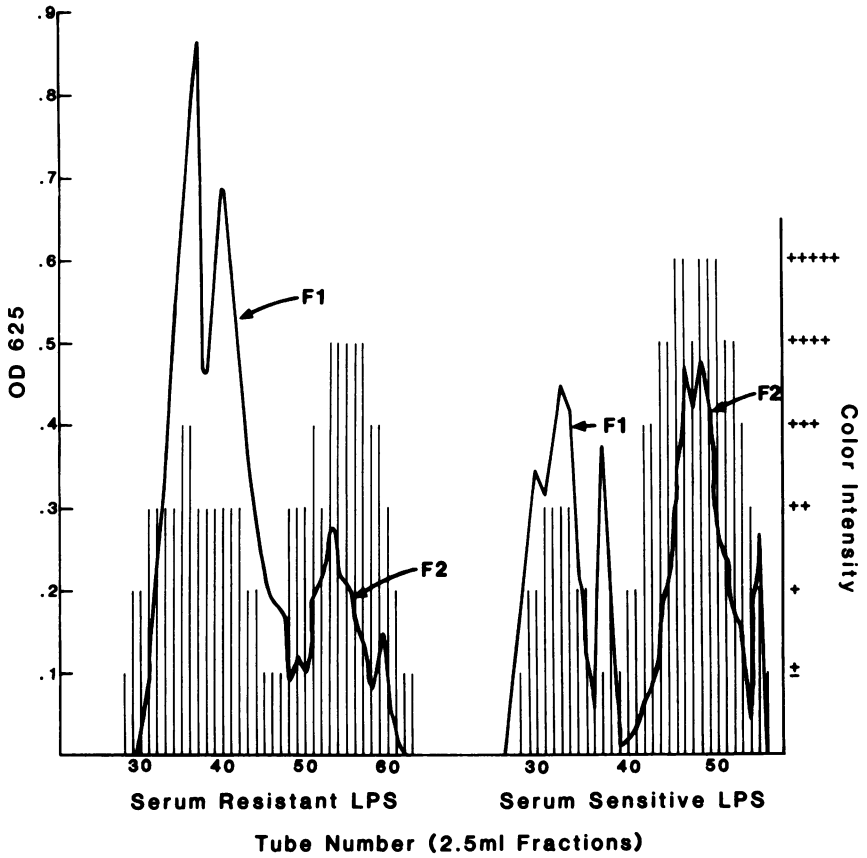
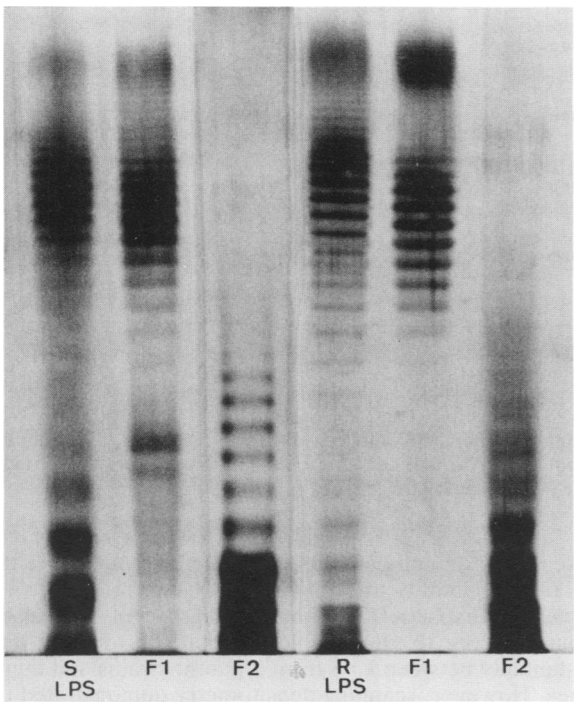


FIG. 4. Fractionation of DOC-disaggregated LPS of serum R and S *E. coli* 227 on Sephadex G-200 and chemical analyses of the higher-molecular-weight (F1) and lower-molecular-weight (F2) fractions for hexose (solid continuous line) and KDO (vertical bars). Core components of LPS from both strains are not shown. OD 625, Optical density at 625 nm.



members of the family *Enterobacteriaceae*, although rough bacilli may be killed by complement components alone (6, 15, 20, 27). The classical and alternative complement systems both participate in serum killing, although the classical pathway induces more rapid bactericidal activity than the alternative pathway (21).

Resistance to NHS is often considered a virulence factor since GNB in the gastrointestinal tract are usually serum S while isolates from clinical infection are usually R (12, 19). Mechanisms responsible for the preponderance of serum resistance among clinical isolates have not been delineated, but these studies demonstrate that serum R clones can be selected from a serum S majority population by the killing of susceptible organisms by serum. Selection of phenotypically stable R populations required serial passage but may occur more rapidly in vivo than under the relatively artificial conditions of these studies. Rapid inactivation of complement components in the medium used at the incubation temperatures required might result in less complete elimination of the S population than in clinical conditions in which there is continuous exposure to serum complement.

Several investigators have hypothesized that differences

FIG. 5. PAGE of LPS and Sephadex G-200 F1 and F2 fractions from serum S and R *E. coli* 227 demonstrating the effectiveness of separation in that the F1 fraction of both strains was made up almost exclusively of higher-molecular-weight subunits, while the F2 fraction was exclusively lower-molecular-weight subunits. Subunits representing core components are partially excluded.

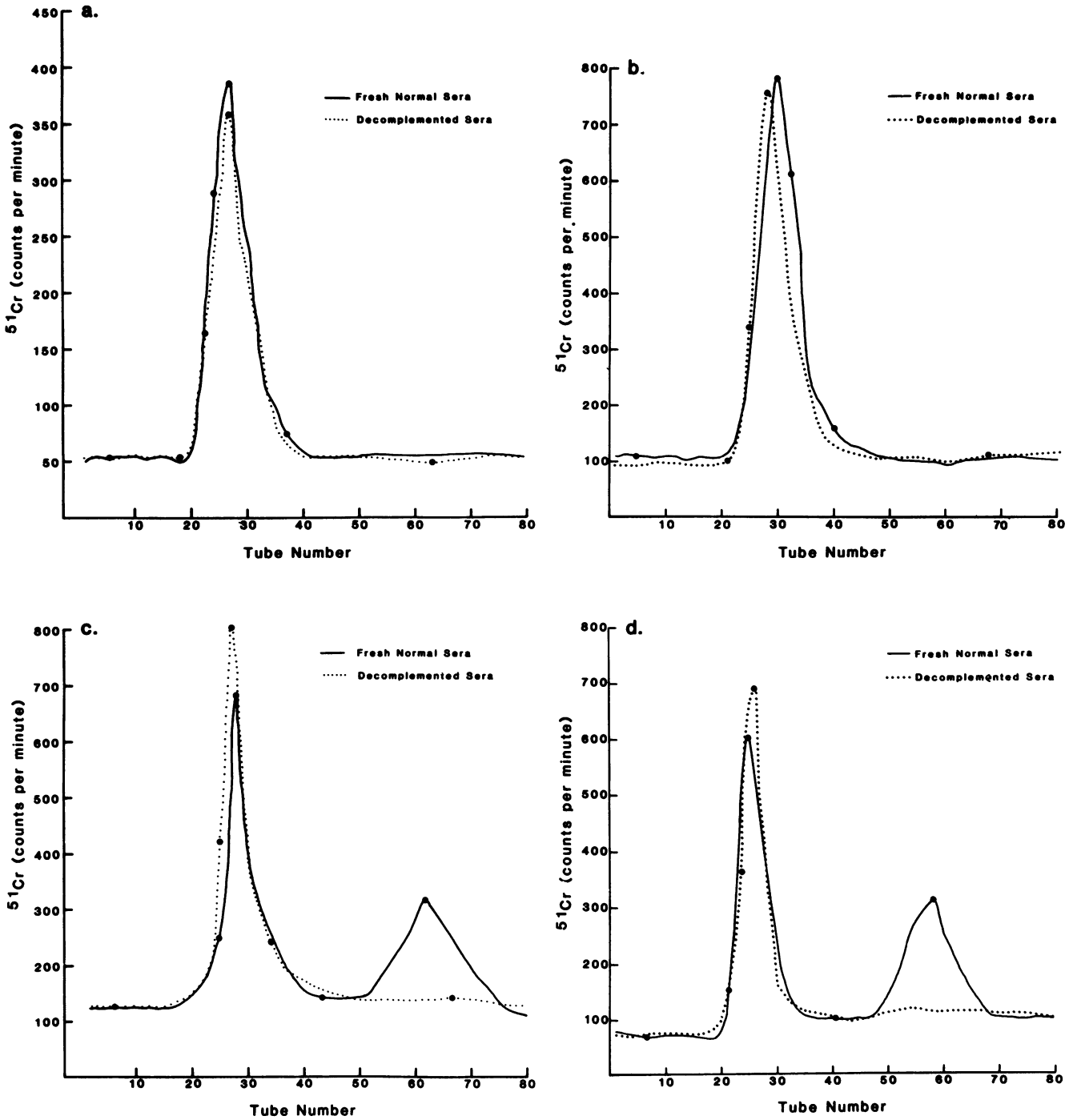


FIG. 6. ^{51}Cr release from liposomes prepared with F1 or F2 fractions of LPS from serum S and R *E. coli* 227 after incubation with NHS. No ^{51}Cr release occurred after incubation with heated serum. Liposomes containing equivalent amounts of the F1 fraction of LPS from both S (a) and R (b) *E. coli* 227 were not lysed, and no free ^{51}Cr was released. In contrast, liposomes containing similar amounts of the F2 fraction from S 227 (c) and R 227 (d) were readily lysed by NHS as evidenced by the second peak of free ^{51}Cr.

between serum S and R bacilli reside in the length of O-antigen chains of LPS (5, 22, 28). Many studies have compared serum R strains with rough mutants (4, 22, 25, 28), whereas these studies compare serum S *E. coli* clinical isolates with derived R clones. These clones were identical to their parents serologically, biochemically, and by antibiotic sensitivity pattern, but LPS from the R clones demonstrated a greater number of longer-chain LPS molecules than LPS of the parent S strain by PAGE.

Overall, hexose/KDO ratios, a measure of the relative amount of core to O-antigenic components, did not differ substantially between LPS from S parent strains and their R clones. However, scanning densitometry demonstrated that LPS from serum R clones contained a higher proportion of

long-chain molecules than LPS from their S parents. Fractionation of disaggregated LPS confirmed that LPS from R clones contained more long-chain subunits than LPS from serum S strains. The longer, more highly O-antigen-substituted LPS subunits contained the same proportion of hexose to KDO (20:1) whether from serum S or R GNB. These differences in relative amounts of subunits of various lengths between LPS from serum S and R GNB do not establish that they, in themselves, are responsible for the variations in serum sensitivity observed. Their significance as determinants of sensitivity or resistance was established, however, by the use of liposomal membranes, simulating bacterial cell walls, which incorporated LPS and ^{51}Cr as a release marker. LPS labeled with ^{3}H from S and R GNB incorporated equally well into liposomes. Entrapment and release studies indicated that larger amounts of LPS were needed for optimal liposome formation than were actually incorporated, with 1 mg of LPS required to form 0.2 ml of liposome suspension while only 12% was incorporated. Liposomes free of excess LPS and incubated with fresh NHS were lysed, releasing approximately 50% of incorporated ^{51}Cr when S LPS was used. Liposomes with no LPS or containing LPS from serum R clones were not lysed by serum, and serum heated to 56°C had no effect on any of the liposomes. Even more striking results were obtained with liposomes incorporating long- or short-chain G-200 fractions of LPS. Liposomes containing equivalent concentrations of the F1 or long-chain LPS subunit fractions from LPS of either S or R GNB behaved like those containing LPS from R GNB in their resistance to lysis by serum. In contrast, liposomes containing F2 or short-chain fractions of LPS from either S or R GNB were readily lysed by serum, similar to liposomes containing LPS from serum S GNB. These findings firmly establish LPS as one reactive site for killing of GNB by NHS. They also demonstrate that resistance to killing by NHS is mediated by the content of longer-chain, high O-substituted LPS subunits. The exact mechanism by which these longer-chain subunits block bactericidal activity has yet to be established but may result from the combination of antibody and complement components at a site too distant from the cell surface to allow the membrane attack complex to damage the cell membrane. Whether this is correct or whether complement activity is blocked in another manner has yet to be determined, but this liposomal model of bacterial cell membranes provides a model that allows more specific delineation of the role of individual bacterial components and their subunits in the serum bactericidal reaction.

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