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Interaction of Gram-Negative Bacteria with the Lysosomal Fraction of Polymorphonuclear Leukocytes

I. Role of Cell Wall Composition of *Salmonella typhimurium*

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Wild-type *Salmonella typhimurium* and cell wall mutants with sequential deficiencies in their cell wall polysaccharide were examined for sensitivity to the bactericidal action of the lysosomal fraction of polymorphonuclear leukocytes. The complete lipopolysaccharide basal core was essential for resistance to the bactericidal action. O-specific side chains of the wild type did not enhance the resistance. Absence of *N*-acetyl glucosamine considerably enhanced sensitivity, whereas absence of other core sugars did not; additional increase in sensitivity was obtained when the heptose phosphate was absent. Under conditions where appropriate supplementation of the medium permitted complete cell wall synthesis, the uridine diphosphate-*gal-4*-epimeraseless mutant regained resistance that was essentially equal to that of the wild type. Cells coated with specific antiserum and nongrowing cells were more resistant than normal growing cells.

Degranulation of phagocytic cells and the release of the lysosomal substances from the lysosomes into the phagocytic vacuoles have indicated that lysosomes play a role in killing and digesting engulfed bacteria (7). Cohn and Hirsch (3) isolated a lysosomal fraction from polymorphonuclear (PMN) leukocytes and showed that it contains hydrolytic enzymes and antibacterial activity. Zeya and Spitznagel (22, 23) found that the antibacterial activity of the PMN leukocyte lysosomal fraction is associated with cationic proteins and later showed that these are confined to a special group of granules poor in hydrolases (25). McRipley and Sabarra (10) described a myeloperoxidase system that is associated with the antibacterial activity of the PMN lysosomes.

Most of the studies on the mechanism of the bactericidal activity of the PMN lysosomes in cell-free extracts have been devoted to the properties of the lysosomes, but have furnished little information on the interaction of the lysosomal fraction with the bacterial surface. The importance of the outer surface of bacteria in host-parasite relationships has been stressed in several studies of different biological systems. The presence of lipopolysaccharide side chains seems to enable the bacteria to resist phagocytosis (12). Loss of sugar components from the O-antigen of enteric bacteria is accompanied by increased sensitivity to bactericidal action of antibody and complement (15). The bactericidal action of immune serum was explained by its interaction with the bacterial cell envelope, involving extensive cell wall damage (5, 21). A rough strain of *Escherichia coli* was found to be sensitive to most of the cationic proteins of the PMN lysosomal fraction, whereas a smooth strain was sensitive to only one of these proteins (24).

The availability of cell wall mutants of *Enterobacteriaceae* with deficiencies in the sequence of polysaccharides in their cell walls (11) enabled us to study the role of bacterial cell wall structure in the interaction with the PMN lysosomal fraction. Moreover, using different phenotypes of the *Salmonella typhimurium* uridine diphosphate (UDP)-*galactose-4*-epimeraseless mutant (after growth under different conditions), we were able to study the effects of the lysosomal fraction on genetically identical cells with deficient and complete cell walls.

MATERIALS AND METHODS

Bacteria and culture conditions. Wild-type *Salmonella typhimurium* and its cell wall mutants with defined deficiencies of sugars in the lipopolysaccharide

TABLE 1. *Description of Salmonella typhimurium strains employed*

Strain	Genotype	Cell wall composition
LT2	S (wild type)	Contains basal core and side chains
TV-119	Ra (RII)	Lacks side chains, but complete basal core
TV-161	Rb (RI)	Lacks side chains and *N*-acetyl glucosamine (GlcNAc)
LT2-M1	Rc	Lacks side chains, GlcNAc, and galactose
SL-1032	Rd	Lacks side chains, GlcNAc, galactose, and glucose
G-30/C21	Re	Lacks side chains, GlcNAc, galactose, glucose and heptose-phosphate

structure were obtained through the courtesy of W. Braun (Rutgers University, Institute of Microbiology, New Brunswick, N.J.). The description of the cell wall composition of the strains is given in Table 1. All the strains were grown in nutrient broth (Difco) at 37 C in a rotatory incubator shaker. To obtain cells in the logarithmic phase, overnight cultures were diluted 10 times with fresh nutrient broth and incubated for an additional 1 to 1.5 hr and harvested at a concentration of 3×10^8 cells/ml by centrifugation ($12,000 \times g$ for 5 min at 4 C). The cell sediment was washed once and suspended in phosphate buffer (0.1 M, *p*H 6). Cell density was adjusted by using a Klett-Summerson colorimeter (filter 42). *S. typhimurium* LT2-M1 (UDP-*galactose*-4-epimeraseless mutant), having a complete cell wall structure when grown on a galactose-supplemented medium (6), was obtained by addition of 0.1% galactose to the nutrient broth-grown culture at the logarithmic phase of growth (1.5×10^8 cells/ml) and by incubation for an additional 40 to 50 min before harvesting.

Preparation of PMN leukocytes and lysomal fraction. High concentrations of PMN leukocytes in the peritoneal exudate of adult male guinea pigs (about 800 g) were induced by intraperitoneal injection of 20 ml of a sterile solution of sodium caseinate (6%, w/v; Eastman Organic Chemicals), a modification of the method described by McRipley and Sbarra (10). Preparation of peritoneal cell exudate and treatment of cells were as described by Cohn and Hirsch (3). An average yield was 5×10^8 cells, 90% of which were PMN. The cell suspension was homogenized in a chilled Potter-Elvehjem homogenizer with a Teflon pestle for 10 min. The homogenate was fractionated as described (3). The lysosomal fraction ($8,000 \times g$) was lysed by suspension in distilled water, then frozen and thawed at least twice to form a crude extract, and stored at −20 C. The protein content of the lysosomal fraction was 50 μg of protein per 10^7 PMN leukocytes, as estimated by the method of Lowry et al. (8).

Reaction mixture. The reaction was carried out in 2 to 5 ml of phosphate buffer (0.1 M, *p*H 6) in a 25-ml siliconized flask at 37 C in a rotatory shaker bath. The reaction mixture contained 10^7 to 3×10^7 bacteria per ml, and the lysosomal fraction was prepared from 0.8×10^7 to 1.6×10^7 leukocytes per ml, unless stated otherwise.

Viable counts of bacteria. Enumeration of the living bacteria in the reaction mixture was made by the microdrop technique of Miles and Misra (14). Duplicate drops (0.2 ml) of serial dilutions of the bacterial suspension were placed on nutrient agar and incubated at 37 C for 18 hr.

Release of 260-nm absorbing material. The amount of acid-soluble 260-nm absorbing material released from bacterial cells during the interaction with the lysosomal fraction was estimated spectrophotometrically in the supernatant fluid ($12,000 \times g$ for 10 min at 4 C) of the reaction mixture. The supernatant fluid was treated with 0.5 M perchloric acid (PCA) for 10 min at 4 C and centrifuged ($14,000 \times g$ for 10 min at 4 C). The 260-nm absorbance of the lysosomal fraction itself was measured, and the value was subtracted from the amount measured for the reaction mixture. The total amount of 260-nm absorbing material in the bacterial suspension after extraction with 0.5 M PCA (15 min at 70 C) was measured.

Antiserum and coating of the bacteria. Preparation of antiserum against *S. typhimurium* LT2-M1 and coating of the bacteria with the specific antiserum were carried out as described (20). The coated bacteria were washed and resuspended in phosphate buffer (0.1 M, *p*H 6).

RESULTS

Sensitivity of the wild type and cell wall mutants of Salmonella typhimurium to the bactericidal action of the lysosomal fraction. The kinetics of the bactericidal action of the lysosomal fraction on the wild type and cell wall mutants are shown in Fig. 1. All the rough mutants with an incomplete basal core (Rb, Rc, Rd, and Re types) were much more sensitive than the wild type (S). The rough mutant, with a complete basal core but lacking the O-specific side chains (Ra type), had resistance similar to that of the wild type.

Comparison of the sensitivity of the LT2-M1 mutant grown under standard growth conditions (Rc type) and in the presence of galactose (S phenotype; LT2-M1-gal) shows that the galactose-grown cells acquired resistance to the lysosomal fraction, and the kinetics of bactericidal action on these cells approached that of the wild type (Fig. 1).

Table 2 summarizes the sensitivity of cell wall mutants and the wild type to the bactericidal action of several concentrations of the lysosomal fraction. All the mutants with an incomplete basal core (Rb, Rc, Rd, and Re types) were sensitive to less concentrated lysosomal fractions. The mutant with complete basal core (Ra) was as resistant as the wild type (S). Rough mutants (Rb, Rc, and Rd types) lacking different sugars

in the basal core had similar sensitivity, whereas the Re type mutant whose basal core consists only of 2-keto-3-deoxyoctonate and lipid was more sensitive than the others. Mutant LT2-M1 not only regained resistance when grown on galactose (LT2-M1-gal), but its level of resistance may even exceed that of the wild type.

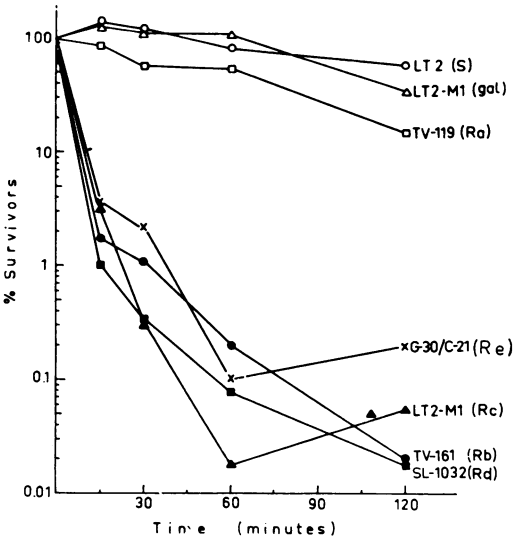

FIG. 1. *Kinetics of the bactericidal action of the lysosomal fraction on wild-type cells and cell wall mutants of Salmonella typhimurium. Bacterial strains are as designated in Table 1; LT2-M1 (gal) (S phenotype) is the LT2-M1 (Rc) strain after growth on galactose. Number of survivors was determined by viable count of bacteria at given times of incubation of the reaction mixture.*

Release of 260-nm absorbing material by the lysosomal fraction from S. typhimurium of different cell wall compositions. The kinetics of the release of 260-nm absorbing material from *S. typhimurium* mutant LT2-M1 are illustrated in Fig. 2. After growth in the presence of galactose, the release of 260-nm absorbing material was reduced. Even when a less concentrated lysosomal fraction was used, the amounts of 260-nm absorbing material released from cells grown in the absence of galactose were larger than those obtained with higher concentrations of lysosomal fraction acting on LT2-M1 cells grown in galactose-supplemented medium.

Effect of antibody coating on the interaction of strain LT2-M1 with the lysosomal fraction. The kinetics of the bactericidal activity of the lysosomal fraction on the mutant LT2-M1 after coating of the bacteria with specific antiserum are shown in Fig. 3. Coating of the bacteria with specific antiserum protected the cells; it diminished the rate of killing observed during the early phase, and led to only a negligible change in the viable count thereafter. Killing continued at a relatively high rate in uncoated cells.

Resistance to the bactericidal effect of the lysosomal fraction was enhanced by increasing the amount of antiserum used (Table 3). Coated bacteria incubated with low concentrations of the lysosomal fraction (prepared from 0.4×10^7 and 0.8×10^7 PMN leukocytes/ml) multiplied; the uncoated cells did not multiply, even when incubated without the lysosomal fraction. This multiplication might have been due to the utilization of lysosomal fraction substances as nutrients by the coated cells.

Effect of the physiological state on the sensi-

TABLE 2. *Sensitivity of Salmonella typhimurium wild type and the cell wall mutants to the bactericidal activity of lysosomal fraction*

Strain	Designation	Concn[a]								PMN ($\times 10^{-7}$) per ml of lysosomal fraction calculated to cause 90% killing
		6.4	3.2	1.6	0.8	0.4	0.16	0.08	0	
LT2-M1 (gal)	S[b]	0.4[c]	140	117	105	80	110		100	6.4
LT2	S	0.8	10	53	118	86	92		100	3.2
TV-119	Ra		4.2	51	90	83	90		100	3.2
TV-161	Rb			0.45	5	40	92		100	0.8
LT2-M1	Rc			0.02	0.43	71	86		100	0.8
SL-1032	Rd			0.07	0.6	36	100		100	0.8
G-30/C21	Re			0.05	0.85	5	49	80	100	0.4

[a] Concentrations of lysosomal fractions are designated as number of PMN leukocytes $\times 10^{-7}$ per milliliter, from which the fractions were prepared.

[b] Phenotype (see Fig. 1).

[c] Values are expressed as the percentage of bacterial cells after 60 min of treatment with different concentrations of lysosomal fraction.

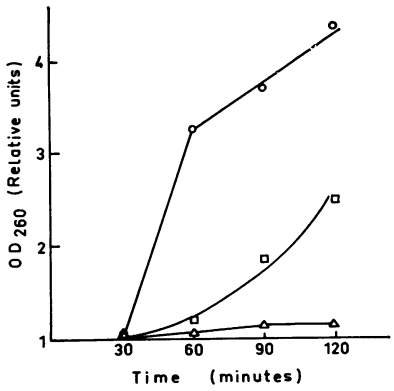

FIG. 2. *Release of 260-nm absorbing material by the lysosomal fraction from Salmonella typhimurium LT2-M1 grown under standard conditions* (□, ○) *and from galactose-grown LT2-M1 cells* (△). *Each milliliter of the reaction mixture contained 3 × 10⁸ bacteria and lysosomal fraction prepared from 7 × 10⁷ PMN* (○, △) *or from 4 × 10⁷ PMN* (□). *Relative units expressed as the ratio of optical density (OD) of supernatant fluid from treated bacteria to OD of supernatant fluid from untreated bacteria.*

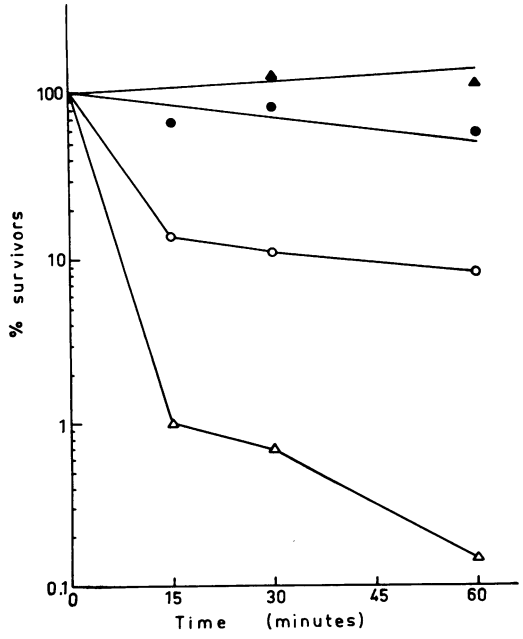

FIG. 3. *Effect of coating Salmonella typhimurium LT2-M1 bacteria with a specific antiserum on the kinetics of bactericidal activity of the lysosomal fraction. Coating was carried out with 0.1 ml of specific antiserum per 3 × 10⁹ bacteria. Coated bacteria treated with lysosomal fraction* (○), *normal bacteria treated with lysosomal fraction* (△), *coated bacteria untreated* (●), *normal bacteria untreated* (▲).

TABLE 3. *Effect of coating of the bacteria with antibody on the bactericidal activity of the lysosomal fraction on Salmonella typhimurium LT2-M1*

Amt of antiserum[a]	Concn					PMN (× 10^{-7}) per ml of lysosomal fraction calculated to cause 50% killing
	1.6	0.8	0.4	0.16	0	
0.0	0.3[b]	0.46	39	100	100	0.4
0.1	7	25	163		100	0.8
0.2	37.5	180	475		100	1.6

[a] Expressed as milliliters per 3 × 10^9 bacteria.
[b] Values are expressed as percentage of bacterial cells with different lysosomal fraction concentrations (see Table 2).

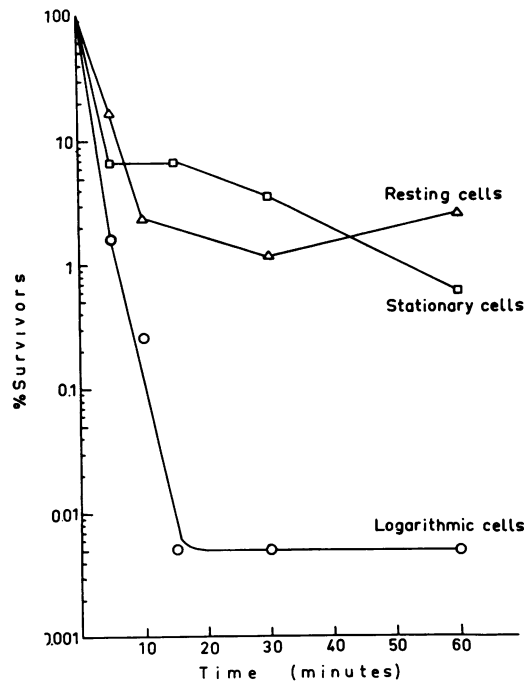

FIG. 4. *Effect of the physiological state of Salmonella typhimurium LT2-M1 on the kinetics of bactericidal activity of the lysosomal fraction. Resting cells were prepared from a culture in the logarithmic phase by washing and incubation in phosphate buffer (0.1 M, pH 6) at room temperature for 150 min.*

tivity of mutant LT2-M1 to the bactericidal action of the lysosomal fraction. The kinetics of killing of LT2-M1 cells at various stages of cultural growth, involving different physiological states, are shown in Fig. 4. During the first phase of incubation (up to 10 min), the rates of killing of stationary-phase and resting cells were similar to

the rate observed for logarithmic-phase cells. The rapid decline in viable count ceased earlier in stationary-phase and resting cells, and therefore the number of survivors of these cells was greater.

DISCUSSION

All the mutants of *S. typhimurium* possessing incomplete polysaccharide core structure of their cell wall (Rb, Rc, Rd, and Re types) were found to be much more sensitive to the bactericidal action of the PMN lysosomal fraction than the smooth wild type. The cell wall mutant possessing a complete basal core, but lacking the O-specific side chains (Ra type), has resistance similar to the wild type. Thus, it appears that the existence of the whole basal core is essential for resistance to the bactericidal action of the PMN lysosomal fraction, whereas the presence of O-specific side chains does not contribute markedly to resistance. The absence of only the terminal *N*-acetyl glucosamine from the basal core (Rb type) is sufficient to enhance sensitivity. Additional deficiencies in glucose and galactose (Rc, Rd, and Re types) result in no additional increases in the sensitivity of the bacteria, whereas the absence of heptose-phosphate in the extreme rough type (Re) causes an additional increase in sensitivity (Table 2).

The enhanced resistance of strain LT2-M1, when transformed from a rough to a smooth phenotype, corroborates the results with cells of different genotypes. The phenotypic transformation was achieved by growing the LT2-M1 mutant in a galactose-supplemented medium, which permits complete cell wall synthesis (6). These two phenotypes, derivable from the same strain, differ also in the amount of 260-nm absorbing material released during incubation with PMN lysosomal fraction.

The drastic reduction in bacterial resistance when only the terminal *N*-acetyl-glucosamine moiety is missing in the cell wall polysaccharide shows its importance as a keystone in the cell wall structure. The terminal position of this sugar in the basal core of the Ra type (9) or of the wild type (16) might cause a steric interference, which protects these cells from the active components of the lysosomal fraction.

It could be postulated that a small degree of degradation of the polysaccharide or lack of terminal *N*-acetyl-glucosamine might suffice to remove the steric hindrance of the bactericidal effect. Indications that this might be the case were obtained in experiments in progress in our laboratory, suggesting that the rate of bacterial lysis by the lysosomal fraction is much more rapid than the disintegration of cell wall lipopolysaccharide by high concentrations of lysosomal fraction. In addition, Malchow et al. (11) did not observe degradation of cell wall polysaccharide during phagocytosis of cell wall mutants of *Salmonella minnesota* by *Dictyostelium discoideum*; similar results were obtained by Rosen et al. (18) with *S. typhimurium* LT2-M1 phagocytized by another species of myxamoeba. Relevant to such considerations is the hypothesis recently advanced by Chedid (2) of a general mechanism of natural immunity to *Enterobacteriaceae*, in which any change in the cell wall structure which unmasks R-antigenic sites permits an initiation of bactericidal interactions with serum factors.

It has been shown that the O-specific side chains are necessary for *E. coli* to resist phagocytosis by PMN leukocytes in vitro and by monocytes in vivo (12). Similar results were found for *S. minnesota* phagocytized by *D. discoideum* (11). It seems that there is a difference between the cell wall components responsible for resistance to the PMN leukocyte lysosomal fraction and the components responsible for resistance to phagocytosis; these different resistances deserve further investigation.

An increased resistance to the bactericidal action of lysosomal fraction was obtained by coating *S. typhimurium* LT2-M1 with a specific antiserum. This effect may be due to a diminished ability of factors in the lysosomal fraction to approach their target as a consequence of steric or electrostatic interference, or both.

Logarithmic-phase cells of *S. typhimurium* LT2-M1 were found to be more sensitive than stationary-phase or resting cells. Similarly, growing cells are known to be more sensitive to the actions of lysozyme and ethylenediaminetetraacetic acid (17), of lysozyme alone (1), and of complement-dependent serum factors (13). This may be because of differences in the cell wall structure which have been observed in certain bacteria at different growth phases or at different growth rates. A decrease in the amount of cell wall components was observed when the growth rate of *Salmonella enteritidis* increased, and this was correlated with variations S to R (4). Also, *Streptococcus faecalis* cells from the stationary phase have larger amounts of cell wall components than do cells in the logarithmic phase (19).

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