

# Interaction of Complement and Polymyxin with Gram-Negative Bacteria

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The interaction of complement and polymyxin with gram-negative bacteria has been investigated by using three strains of *Salmonella typhimurium* in an attempt to determine the loci of complement action. It has been shown that the bactericidal activities of complement and polymyxin towards a smooth gram-negative organism are similarly affected by various components of the bactericidal test system. Further, complement and polymyxin have been shown to act synergistically in the bactericidal event. Evidence is presented which suggests that each agent produces lesions in the outer membrane of gram-negative bacteria, allowing lysozyme to interact with its glycopeptide substrate. An attack on the inner cytoplasmic membrane follows, since cell respiration is rapidly inhibited and this membrane becomes sufficiently disorganized to permit massive leakage of  $\beta$ -galactosidase from the cytoplasm of the target cells.

In earlier studies of serum bactericidal activity, it was shown that certain serum-resistant, smooth gram-negative organisms could be converted to serum sensitivity under environmental conditions which had a disruptive effect on their outer lipopolysaccharide layer (19, 20). Thus, resistance to the bactericidal effect of specific antibody and complement is probably due to the protective function of the thick lipopolysaccharide layer as was originally suggested by Wardlaw (26) and by Muschel (12). Although the mechanism of this cytotoxic action is not as yet well understood, both the outer (20) and inner membrane (28) of the gram-negative bacteria are thought to be damaged, and it appears probable that damage to the inner cytoplasmic membrane constitutes the lethal event (19).

Consequently, it was of interest to compare the interaction of complement and polymyxin with gram-negative organisms since polymyxin is also known to attack both the outer (27) and inner (24) membranes of gram-negative organisms.

The susceptibility of bacteria to the cytotoxic action of polymyxin and serum complement have certain features in common. The bactericidal activity of both complement and polymyxin is limited by the permeability barrier of the outer cell wall, and protoplasts of serum- or polymyxin-resistant organism are susceptible to the lytic activity of complement (15) and poly-

myxin (24), respectively. Further, magnesium ions, which contribute to the structural integrity of the lipopolysaccharide of gram-negative organisms, antagonize the bactericidal action of both complement (14, 19) and polymyxin (17) on smooth gram-negative organisms.

In this paper, we report further comparisons of the complement and polymyxin systems, and demonstrate their synergistic activity on a smooth strain of the gram-negative organism *Salmonella typhimurium*.

## MATERIALS AND METHODS

**Strains, antiserum, and bactericidal assays.** A previous report describes the strains of *S. typhimurium* C5 and M206, antiserum, and the complement-mediated bactericidal assays used (19). *S. typhimurium* LT2 F'*lac* carries the F'13 *lac* operon of *Escherichia coli* K-12 and is consequently inducible for  $\beta$ -galactosidase. This strain was kindly sent to us by R. F. Goldberger, National Institute of Health, Bethesda. Bactericidal assays involving polymyxin were carried out as for complement-mediated bactericidal assays by dilution of polymyxin from a stock solution of 1 mg/ml in physiological saline which was stable at 4 C for at least 1 week. Polymyxin B sulfate was obtained from Calbiochem, Los Angeles, California.

**Release of  $\beta$ -galactosidase.** *S. typhimurium* LT2 F'*lac* was grown to about  $5 \times 10^7$  cells/ml in nutrient broth with mild aeration and methyl- $\beta$ -D-thiogalactopyranoside (Calbiochem) was added to  $5 \times 10^{-4}$  M. With a further period of 1.5 hr of growth, the induced cells were centrifuged and suspended in tris(hydroxymethyl)aminomethane (Tris)-

chloride buffer (0.1 M, pH 7.4) to  $5 \times 10^8$  cells/ml at 37 C for 30 min in the presence of a 1:500 dilution of rabbit antiserum. Ethylenediaminetetraacetic acid was added to  $6 \times 10^{-4}$  M and further incubated for 10 min. The cells were centrifuged and resuspended in Tris-chloride buffer to  $5 \times 10^8$  cells/ml, and 7.5-ml samples were dispersed into 20-ml bottles. A 0.5-ml amount of reagents was added, and the cells were incubated at 37 C and sampled for surviving organisms and release of  $\beta$ -galactosidase. Assays for released intracellular  $\beta$ -galactosidase were carried out by dispersing 0.5 ml, in duplicate, into an equal volume of ice-cold 0.1 M phosphate buffer, pH 7.4, with 0.04 M  $Mg^{2+}$ . The samples were centrifuged for 10 min at  $5,000 \times g$  in the cold, and 0.5 ml of the supernatant fluids were dispersed into 1.5 ml of phosphate buffer and 0.5 ml of the enzyme substrate, 0-nitro-phenyl- $\beta$ -D-galactopyranoside (Calbiochem, Los Angeles, Calif.), to 250  $\mu$ g/ml. The tubes were incubated for 15 min at 37 C, and the reaction was stopped by adding 1 ml of 1.0 M sodium carbonate solution. Total enzyme content was determined in toluenized cells. The color reaction was read at 420 nm.

**Radioactive labeling of glycopeptide.** A previous report (20) describes the radioactive labeling of glycopeptide and the assay procedure for measuring acid-insoluble radioactivity.

**Oxygen uptake.** Oxygen uptake was measured by using a YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Cells growing exponentially in nutrient broth were washed and resuspended in diluent at about  $5 \times 10^7$  cells/ml, and rabbit antibody at a dilution of 1:1,000 was preabsorbed for 30 min at 37 C. A 2.5-ml amount of the cells was dispersed into oxygen monitor chambers, guinea pig serum or polymyxin was added, and the rate of oxygen consumption was determined.

**Synergistic action of guinea pig serum and polymyxin.** Bactericidal tubes were set up containing combinations of guinea pig serum dilutions from 20 to 1% and polymyxin from 1  $\mu$ g/ml to 0.05  $\mu$ g/ml in physiological saline, and rabbit antiserum at a dilution of 1:1,000, with controls of polymyxin and complement alone. Bacteria were added to approximately  $10^4$  cells/ml, and the percentage of survivors was determined as described in a previous report (19).

## RESULTS

**Bactericidal activity of polymyxin and antibody-complement in various salt solutions.** The bactericidal activities of antibody plus complement, and of polymyxin against the smooth C5 strain of *S. typhimurium* and the semi-rough M206 strain, were compared in various test media (Table 1).

C5 was relatively insensitive to both complement and polymyxin in physiological saline but was markedly sensitive to either agent in Tris-chloride buffer. However, the addition of

$Mg^{2+}$  (0.004 M) to either saline or Tris-chloride medium inhibited killing of C5 by both polymyxin and complement.

The semi-rough M206 strain which was quite sensitive to both complement and polymyxin in saline medium showed still greater sensitivity in Tris-chloride. Unlike the smooth C5 strain, M206 was afforded little protection by  $Mg^{2+}$  when treated with polymyxin, and the addition of  $Mg^{2+}$  positively enhanced the bactericidal activity of antibody plus complement against M206.

The data presented in Table 2 emphasize differences in the protective action of  $Mg^{2+}$  on the strains C5 and M206 in saline media. Both strains showed greater resistance to the bactericidal action of polymyxin in the presence of increasing concentrations of  $Mg^{2+}$ . This divalent cation also inhibited the bactericidal action of antibody and complement towards the smooth C5 strain.

However, concentrations of up to 0.004 M  $Mg^{2+}$  clearly potentiated the bactericidal action of complement towards the semi-rough M206 strain.

**Minimal inhibitory polymyxin concentration.** Experiments were carried out in order to determine the minimal number of polymyxin molecules per cell that would inhibit growth of *S. typhimurium* C5 and M206. The semi-rough strain M206, being more sensitive to the action of polymyxin (Table 1), was also shown to require approximately a 10-fold decreased polymyxin-to-cell ratio to inhibit growth in nutrient broth (Table 3). Assuming a molecular weight of 1,200 for polymyxin and a 100% absorption of the antibiotic, bacteriostasis was observed at a polymyxin-to-cell ratio of approximately  $20 \times 10^5$  for C5 and  $1.25 \times 10^5$  for M206. Equivalent rates of kill were obtained for both strains but at widely differing polymyxin-to-cell ratios.

**Synergistic action of complement and polymyxin.** The foregoing data suggested that the smooth C5 strain of *S. typhimurium*, owing to its relative resistance to both the action of polymyxin and complement, would provide a suitable system for investigating any synergistic action between these two agents. In preliminary experiments, it was found that the addition of low levels of polymyxin to guinea pig serum and antibody greatly enhanced the bactericidal activity against C5 in saline. Figure 1 shows the bactericidal activity of varying guinea pig serum concentrations and varying levels of polymyxin against *S. typhimurium* C5 in physiological saline. The points of the isobologram were plotted as the level of polymyxin in micrograms per milliliter required

TABLE 1. Bactericidal action of antibody and complement and polymyxin B sulfate against *S. typhimurium* C5 and M206 in various salt solutions<sup>a</sup>

Salt solution, pH 7.4	% Inoculum surviving after incubation for 45 min in:					
	Salt solution (C5) <sup>b</sup>	Salt solution + C + Ab (C5)	Salt solution + POL (C5)	Salt solution (M206)	Salt solution + C + Ab (M206)	Salt solution + POL (M206)
0.9% Sodium chloride	>100	75	80	90	12	10
0.9% Sodium chloride + 0.004 M Mg <sup>2+</sup>	>100	>100	>100	>100	4	42
0.1 M Tris-chloride	>100	4	3	85	<1	3
0.1 M Tris-chloride + 0.004 M Mg <sup>2+</sup>	>100	90	>100	90	<1	8

<sup>a</sup> Bacteria were incubated at 37 C in the presence of 1 µg of polymyxin/ml or in the immune system which consisted of rabbit antiserum at 1:2,000 (Ab) and guinea pig serum (C) which was used at a 1:10 dilution for C5 and 1:100 for M206.

<sup>b</sup> Numbers in parentheses indicate strain numbers.

to kill greater than 50% of the inoculum in 60 min in the presence of a given level of guinea pig serum and a fixed amount of antibody. At the highest concentrations used in this system, neither polymyxin nor antibody-complement gave more than 20% kill, and heated guinea pig serum did not enhance kill in the presence of polymyxin. The deviation from linearity shown in Fig. 1 indicates that polymyxin and complement are synergistic in their activity against C5 in saline.

**Kinetics of glycopeptide degradation.** Having determined that polymyxin and complement act synergistically in the gross bactericidal event, we examined the interaction of these two agents, both separately and in combination, with the outer membrane of C5 cells. We assessed lesion formation in the outer membrane in terms of the ability of lysozyme to penetrate the outer wall and to degrade the underlying glycopeptide layer. The data presented in Fig. 2 show the rate of lysozyme depolymerization of wall glycopeptide when C5 cells suspended in either Tris or physiological saline were treated with polymyxin and complement, alone or in combination, in the presence of lysozyme.

When either polymyxin or complement were used separately, only in Tris medium was there sufficient disruption of the outer lipopolysaccharide envelope to allow lysozyme to penetrate to its substrate. This did not occur in physiological saline unless polymyxin and complement were both present in the reaction mixture (Fig. 2A). Although no lysozyme sensitivity could be demonstrated in C5 cells suspended in saline and treated with polymyxin (10 µg/ml), the cells were rapidly killed. When C5 cells suspended in saline were treated with comple-

TABLE 2. Magnesium ion protection of *S. typhimurium* strains C5 and M206 from the bactericidal activity of polymyxin B sulfate and the antibody-complement system in 0.9% saline solution<sup>a</sup>

Molarity Mg <sup>2+</sup>	% Inoculum surviving after incubation for 45 min in:			
	Polymyxin (1 µg/ml)		Antibody + complement	
	C5	M206	C5	M206
	80	10	75	12
0.001	84	17	>100	<1
0.002	90	30	>100	1
0.004	>100	42	>100	4
0.008	>100	80	>100	22

<sup>a</sup> Bacteria were incubated for 45 min in 0.9% saline containing various levels of magnesium sulfate. Polymyxin was added to 1.0 µg/ml, and guinea pig serum was added to final dilutions of 1:10 and 1:100 for *S. typhimurium* C5 and M206, respectively. Rabbit antiserum was used at a final dilution of 1:2,000.

ment, lysozyme was unable to interact with the C5 glycopeptide (Fig. 2B), and no significant kill of C5 was observed.

Thus, the synergistic action of polymyxin and complement in disrupting the outer membrane of C5 cells can be detected when the cells are suspended in physiological saline. However, whereas complement must make significant breaches in the outer membrane (sufficient to allow the penetration of lysozyme, molecular weight ~ 14,000) if the C5 is to suffer lethal damage, polymyxin can penetrate the outer

TABLE 3. Bactericidal action of polymyxin B sulfate against *S. typhimurium* C5 and M206 at increasing ratio of polymyxin molecules to bacterial cells<sup>a</sup>

Strain	Polymyxin/cell ratio (X10 <sup>5</sup> )	% Inoculum surviving at time (min)			
		12	24	40	60
C5					240
	20				130
	40	92	42	33	44
	160	90	33	10	12
	640	90	11	7	3
M206	—				220
	1.25				100
	2.5	100	100	90	70
	5	100	89	44	40
	10	80	53	18	10
	20	1.5	0	0	0

<sup>a</sup> Bacteria were incubated at 37 C in broth containing 1 µg of polymyxin/ml. Cell numbers were adjusted to give the desired polymyxin-to-cell ratio in a final volume of 1.0 ml.

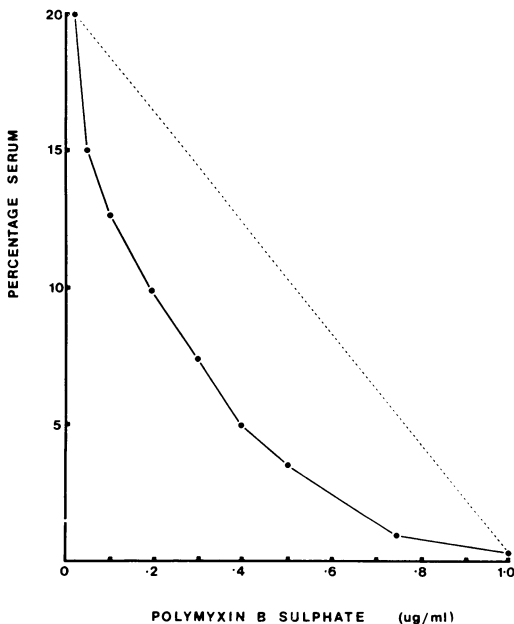


FIG. 1. Isobologram of the bactericidal activity of polymyxin and guinea pig serum and antibody on *S. typhimurium* C5. Each point represents the minimal bactericidal concentration for polymyxin required to kill 50% of the inoculum in saline, in the presence of the indicated concentration of guinea pig serum and 1:10,000 rabbit anti-C5 serum. The guinea pig serum was absorbed with bentonite to remove serum lysozyme.

membrane and kill the cell in the absence of such lesions.

**Inhibition of cell respiration.** The marked sensitivity of C5 cells to either polymyxin (1 µg/ml) or complement (1:10) in Tris-chloride solution, which was observed in bactericidal tests (Table 1), was also apparent when the effect of these agents on cell respiration was used to assess damage to the cytoplasmic membrane (Fig. 3).

With either agent, the respiration of C5 cells suspended in Tris was affected within 4 min and was completely arrested within 20 min. When C5 cells were suspended in physiological saline and treated with antibody and complement, their respiration was not impaired although polymyxin began to affect respiration after 10 min. However, the very rapid inhibition of respiration observed when C5 cells suspended in physiological saline were treated with both complement and polymyxin points to the synergistic action of these two agents.

This observation, together with the data presented in Fig. 4 which showed that complement, in the absence of serum lysozyme, was still able to cause a rapid inhibition of bacterial cell respiration, suggests that the complement system acts directly on the cytoplasmic membrane. Indeed, serum lysozyme appeared to play little part in this particular process since the addition of extraneous lysozyme did not replace the complement activity which was lost on bentonite adsorption (Fig. 4).

**Release of β-galactosidase.** To further investigate the membrane damage produced by polymyxin and complement, we examined the effect of these agents on *S. typhimurium* LT2 F<sup>1</sup>lac in which we could induce high levels of the readily assayable enzyme β-galactosidase as a cytoplasmic component.

Although the LT2 F<sup>1</sup>lac bacteria were rapidly killed by complement or polymyxin in the absence of lysozyme (Fig. 6), negligible release of β-galactosidase was observed in the absence of lysozyme (Fig. 5). Consequently, the release of this high-molecular-weight enzyme (~400,000) by complement or polymyxin appears dependent on the concomitant depolymerization of the glycopeptide sheath.

Thus, although polymyxin and complement may cause sufficient damage to the cytoplasmic membrane to allow cytoplasmic leakage of β-galactosidase, this large enzyme may still be retained if the glycopeptide sheath is not damaged by lysozyme. Alternatively, the release of β-galactosidase in the presence of lysozyme may result from disruption of the cells by internal

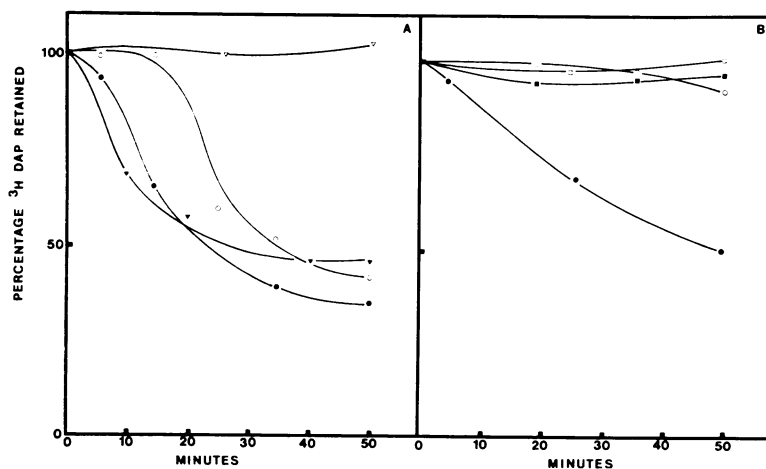


FIG. 2. Kinetics of lysozyme degradation of cell wall glycopeptide of *S. typhimurium* C5 suspended in Tris or in saline. Bacteria were grown in nutrient broth in the presence of  $^3\text{H}$ -DAP (diaminopimelic acid), washed in physiological saline at 37 C, and resuspended in physiological saline at about  $1 \times 10^9$  cells/ml with rabbit antiserum (1:500). After 20 min of incubation at 37 C, the cells were washed with physiological saline and resuspended in either Tris (0.1 M; pH 7.4) or in physiological saline at 37 C and treated with any or all of the following reagents: guinea pig serum (1:10) (this was bentonite adsorbed, 10 mg/ml for 5 min at 5 C), lysozyme at 5  $\mu\text{g}/\text{ml}$ , and polymyxin B sulfate at 10  $\mu\text{g}/\text{ml}$ . Symbols: A:  $\nabla$ , saline, polyximin, and lysozyme;  $\circ$ , saline, polyximin, lysozyme, and guinea pig serum;  $\blacktriangledown$ , Tris, polyximin, and lysozyme;  $\bullet$ , Tris, polyximin, complement, and lysozyme. B:  $\square$ , saline and guinea pig serum;  $\blacksquare$ , Tris and guinea pig serum;  $\circ$ , saline, lysozyme, and guinea pig serum;  $\bullet$ , Tris, lysozyme, and guinea pig serum.

osmotic forces following damage to the supporting glycopeptide sheath. However, polymyxin and complement both seem able to cause very significant damage to the cytoplasmic membrane since  $\beta$ -galactosidase was still released from cells in the presence of concentrations of sucrose which have been shown to stabilize spheroplasts (10). For example, induced cells suspended in 0.5 M sucrose still released their total enzyme content when treated with polymyxin (10  $\mu\text{g}/\text{ml}$ ) and lysozyme (5  $\mu\text{g}/\text{ml}$ ). Similar experiments carried out in 0.3 M sucrose, by using complement (1:10) and lysozyme (5  $\mu\text{g}/\text{ml}$ ), released about 50% of total enzyme content. A lower sucrose concentration was necessitated in the latter experiments since high concentrations of sucrose are anticomplementary (16).

## DISCUSSION

The permeability of gram-negative cells to various solute molecules is not entirely determined by the cytoplasmic membrane. In certain instances, at least, the outer cell wall components may function in permeability control since they restrict the penetration of relatively large molecules, such as actinomycin, into the cell (8, 12) and the loss from the cell of certain enzymes, such as alkaline phosphatase (3), which are located between the outer layers of the

cell wall and the cytoplasmic membrane. The lipopolysaccharide of gram-negative bacteria appears to be a major structural component of the outer membrane system for the following reasons: (i) treatments which grossly affect the structural integrity of the lipopolysaccharide layer remove the restraint on the passage of some relatively large molecules (3, 8, 13, 18); (ii) serum complement and antibody produce lesions in lipopolysaccharide (2) and release membranous spherules from the outer wall of a smooth *E. coli* strain (28); (iii) complement activated by antibody directed against polysaccharide determinants in the lipopolysaccharide has been shown to modify the barrier function of the outer cell wall thereby allowing lysozyme to penetrate and interact with its glycopeptide substrate (5, 20) and alkaline phosphatase to leak from the periplasmic space (3); (iv) certain smooth strains of gram-negative bacteria, which are normally resistant to complement-mediated damage, can be sensitized under environmental conditions which produce a more open structure in smooth lipopolysaccharide (20). Such conformational changes presumably make the lipopolysaccharide more susceptible to attack, possibly by uncovering the phospholipid core material which may be the target of complement action (28).

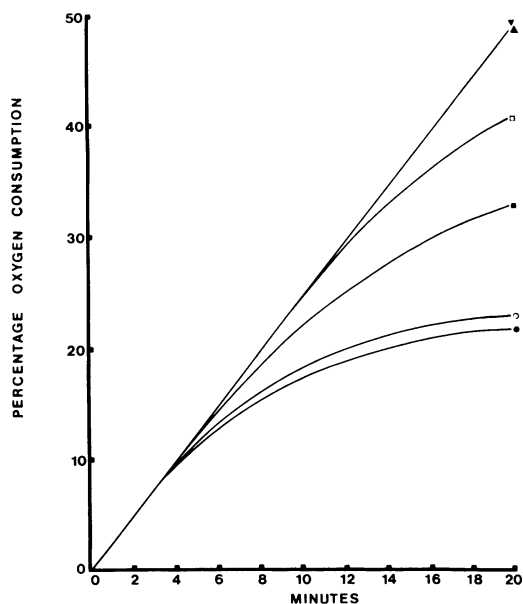


FIG. 3. Inhibition of respiration of *S. typhimurium* C5 by polyxymxin and normal guinea pig serum. Bacteria were preabsorbed with rabbit antibody (1:1,000) for 15 min at 37 C and normal guinea pig serum (1:10) or polyxymxin (1  $\mu$ g/ml) was added. Symbols:  $\nabla$ , Tris suspension and heated guinea pig serum;  $\blacktriangle$ , saline suspension and guinea pig serum;  $\square$ , saline suspension and polyxymxin;  $\circ$ , Tris suspension and polyxymxin;  $\bullet$ , Tris suspension and guinea pig serum;  $\blacksquare$ , saline suspension, guinea pig serum, and polyxymxin.

It therefore seems probable that the outer components of the gram-negative cell wall constitute a permeability control system, in which the lipopolysaccharide component plays a major role, and that damage to the lipopolysaccharide destroys this barrier function. Thus, in the complement-mediated bactericidal event, the breaching of the outer membrane would result from complement-induced lesions in the lipopolysaccharide layer. It also seems likely that complement subsequently interacts with the inner cytoplasmic membrane, again attacking a phospholipid component, as suggested by Wilson and Spitznagel (28), and that it is this interaction which constitutes the lethal event. There is evidence that this may be so since complement destroys spheroplasts (15) and L-phase variants (9), very rapidly inhibits respiration of sensitive cells (19) even in the absence of lysozyme (Fig. 4), and, again in the absence of lysozyme, damages the cytoplasmic membrane releasing micro-molecular cytoplasmic components (23).

Since polyxymxin is known to interact with the outer (22, 24) and inner membrane system (4,

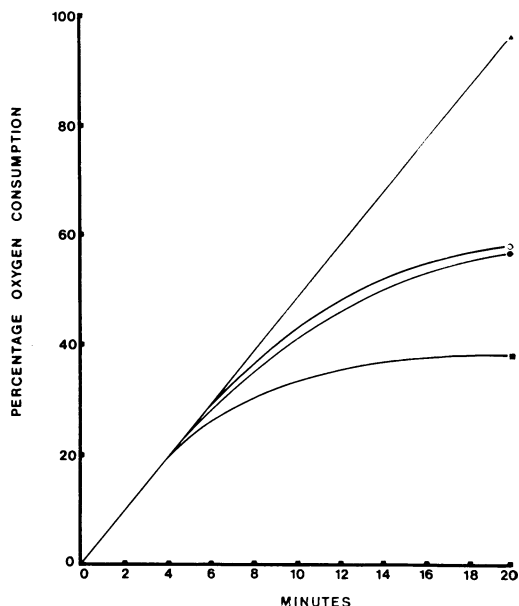


FIG. 4. Inhibition of respiration of *S. typhimurium* M206 by 1:10 normal and bentonite-absorbed guinea pig serum in the presence of 1:3,000 rabbit antiserum, suspended in physiological saline. Symbols:  $\blacktriangle$ , heated guinea pig serum;  $\circ$ , bentonite-absorbed guinea pig serum and 30  $\mu$ g of lysozyme/ml;  $\bullet$ , bentonite-absorbed guinea pig serum;  $\blacksquare$ , normal guinea pig serum.

6), we have attempted to obtain further evidence for this proposed sequence of events by comparing the interaction of complement and polyxymxin with gram-negative bacteria. We have shown that the environmental conditions that favor the killing of the smooth gram-negative strain *S. typhimurium* C5 by antibody and complement also make this strain more sensitive to polyxymxin. Although most gram-negative bacteria are sensitive to polyxymxin, the minimum inhibitory concentration for growth varies and is probably a reflection of both growth conditions and the nature of the outer cell wall which determines the penetration of the antibiotic to the inner cytoplasmic membrane. For example, the marked sensitivity of the semi-rough M206 strain to polyxymxin in physiological saline, compared to the resistance of the smooth C5 under the same conditions (Table 1), is probably a function of cell wall permeability to the antibiotic, since the semi-rough strain possesses only about half as much lipopolysaccharide as the more resistant C5 strain (1). Further, inhibition of growth of M206 occurred at a ratio of about  $2.5 \times 10^5$  molecules of polyxymxin per cell, but with the smooth C5 strain the polyxymxin cell ratio for inhibition of growth was about 10

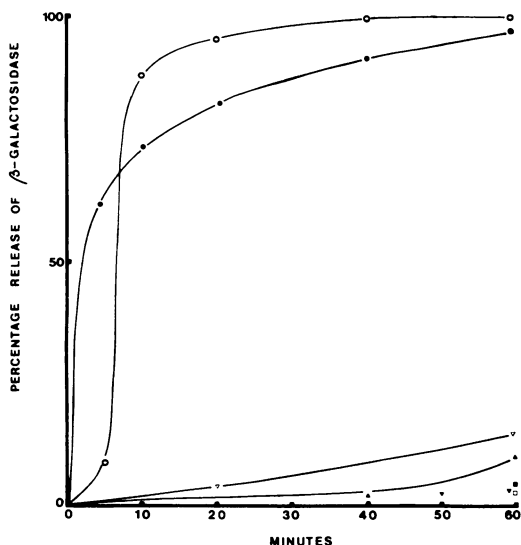


FIG. 5. Kinetics of release of  $\beta$ -galactosidase from *S. typhimurium* LT2 F'lac. Bacteria induced for  $\beta$ -galactosidase were suspended in Tris-chloride (0.1 M, pH 7.4) in the presence of rabbit antiserum (1:500) for 30 min at 37 C. EDTA was added to a final concentration of 200  $\mu$ g/ml and incubated for a further 10 min at 37 C. The cells were centrifuged and resuspended in Tris-chloride buffer and the reagents were added. Guinea pig serum (1:10) was bentonite-absorbed, lysozyme was added to 5  $\mu$ g/ml, and polymyxin to 10  $\mu$ g/ml. Symbols: ○, guinea pig serum and lysozyme; ●, polymyxin and lysozyme; ▲, polymyxin; ▼, lysozyme; ■, polymyxin and guinea pig serum; □, guinea pig serum; ▽, Tris-chloride buffer.

times greater (Table 3). Presumably, the contiguous coat of smooth lipopolysaccharide in the C5 strain hinders effective binding of this cationic antibiotic by masking anionic groupings (presumably phosphate) in the lipid moiety of the outer cell membrane (4, 17, 25), which are more readily available in the semi-rough M206 strain.

Restriction by smooth lipopolysaccharide of the bactericidal action of both polymyxin and complement is again implied by their separate effects on the C5 strain (Table 1). This strain is relatively resistant to both agents in physiological saline, but in 0.1 M Tris, which causes smooth lipopolysaccharide to assume a more open conformation (20), C5 shows comparable increases in sensitivity to each bactericidal agent, becoming as sensitive as the semi-rough M206 strain. Further,  $Mg^{2+}$ , which restores the structural integrity of smooth lipopolysaccharide (18), inhibits Tris sensitizations of the smooth C5 strain to both polymyxin and complement. In contrast, the semi-rough M206 strain, in which

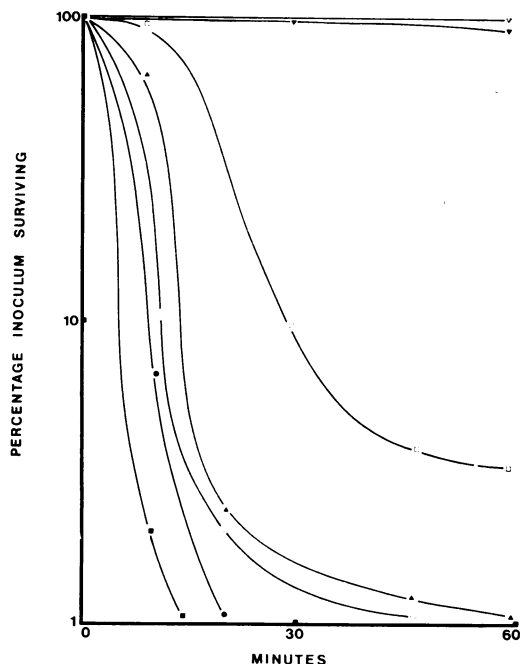


FIG. 6. Bactericidal activity of complement and polymyxin on *S. typhimurium* LT2 F'lac in Tris-chloride buffer. Bacteria were pretreated as before (Fig. 5) and diluted to about  $10^4$  cells/ml into bactericidal tubes. Bentonite-absorbed guinea pig serum was added (1:10), lysozyme to 5  $\mu$ g/ml, and polymyxin to 10  $\mu$ g/ml. Symbols: ▽, Tris-chloride buffer; ▼, lysozyme; □, guinea pig serum; ▲, polymyxin; ○, guinea pig serum and lysozyme; ■, guinea pig serum and polymyxin; ●, lysozyme and polymyxin.

the lipid core is exposed (7), showed very marked sensitivity to both polymyxin and complement in physiological saline and this was not appreciably reversed by  $Mg^{2+}$  (Table 2). However, the data in Table 2 do show that  $Mg^{2+}$  has a greater inhibitory effect on polymyxin than on complement when each is interacted with the semi-rough M206 strain due possibly to two factors: (i) that  $Mg^{2+}$  directly interferes with interactions between the  $NH_3^+$  group of polymyxin and negatively charged phosphate groups in the lipopolysaccharide lipid core which are thought to be responsible for the increased permeability of the outer membrane (22, 25); (ii) that at relatively low concentrations,  $Mg^{2+}$  potentiates complement activity (9).

So far, these data suggest that both the amount of lipopolysaccharide carried by the cells and its conformation are important factors in determining whether polymyxin or complement can effect a cell lesion. A more direct assessment of outer membrane damage was

obtained in terms of the ability of lysozyme to penetrate the outer cell wall and degrade the underlying glycopeptide layer. We found (Fig. 2) that Tris ions clearly facilitated damaging interactions of both polymyxin and complement with the outer membrane of the smooth C5 strain. Such damage was rather more pronounced when the two agents were used in combination. When interacted with C5 in physiological saline, however, neither polymyxin nor complement alone appeared to produce significant outer membrane damage, but when these agents were used in combination in this medium the barrier properties of the outer membrane of C5 were very significantly affected, as lysozyme was then able to reach its glycopeptide substrate. Thus, the synergistic action of polymyxin and complement, which was observed in terms of their combined bactericidal effect on C5 (Fig. 1) and LT2 (Fig. 6), can be accounted for, in part at least, by their combined effect on the outer membrane structure.

Our data also imply that complement attacks the cytoplasmic or inner membrane. Firstly, in Tris buffer, complement caused the same rapid inhibition of respiration of C5 cells that was observed with the membrane-specific antibiotic polymyxin (Fig. 3). Further, guinea pig serum, from which the lysozyme had been removed, rapidly inhibited the respiration of M206 cells in saline (Fig. 4). These observations suggest that complement exerts a direct effect on the cytoplasmic membrane which results in a rapid loss of at least some of the cytoplasmic components involved in respiratory function. Whether or not this damage is sufficiently great to allow relatively large molecules to leak from the cytoplasm is in some doubt, as neither complement nor polymyxin caused the release of  $\beta$ -galactosidase from LT2 *F'*lac cells in the absence of lysozyme (Fig. 5). However, this could well be due to the retention of this high-molecular-weight enzyme by the glycopeptide sheath which does not degrade with either agent in the absence of lysozyme (Fig. 2A and 2B), since Wilson and Spitznagel (28) were unable to obtain release of macromolecular constituents from cells treated with antibody and serum complement from which the lysozyme had been removed. Further, we have found that chloroform does not cause appreciable release of  $\beta$ -galactosidase activity from *F'*lac cells in the presence of Tris buffer. These observations suggest that the high-molecular-weight, intracellular enzyme  $\beta$ -galactosidase can be retained by the glycopeptide layer under conditions where there is gross damage to the inner cytoplasmic membrane. That complement and polymyxin

may each cause gross damage to the cytoplasmic membrane is afforded by our observations that high sucrose molarities did not prevent the leakage of  $\beta$ -galactosidase from the cells suspended in Tris buffer and treated with polymyxin in the presence of lysozyme, whereas complement (which is partially inactivated by sucrose (16)) and lysozyme released approximately 50% of  $\beta$ -galactosidase activity in the presence of 0.3 M sucrose. Thus, complement and polymyxin may each produce relatively large holes in the cytoplasmic membrane, as, in the presence of these high concentrations of sucrose, one would not expect this inner membrane to suffer internal osmotic disruption even though deprived of the external support of the glycopeptide layer.

Thus, there seems to be reasonable evidence that the mode of action of complement and polymyxin are very similar. Each attacks the outer membrane structure of gram-negative bacteria and act synergistically at this locus, at least. Further, the permeability changes in the outer membrane induced by either agent in Tris buffer allow lysozyme to interact with and disaggregate the glycopeptide layer (Fig. 2), and, hence, the bactericidal effect of these agents is potentiated by lysozyme. However, complement, like polymyxin, can kill gram-negative bacteria and rapidly inhibit their respiratory function in the absence of lysozyme. Therefore, it seems very probable that complement acts similarly to polymyxin in that it produces lethal lesions directly in the cytoplasmic membrane of sensitive cells.

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