EFFECT OF POLYMYXIN ON THE LYSIS OF NEISSERIA CATARRHALIS BY LYSOZYME

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In a previous communication (Warren et al., 1955) it was noted that gram-negative bacteria pretreated with heat or acetone were lysed by lysozyme. Since the bacteria studied were not sensitive to the enzyme when conventional methods were used, the possibility was considered that this pretreatment made the substrate in the cell more accessible to the action of the lysozyme by the removal or destruction of a barrier constituent which normally prevents the enzyme from attacking its specific substrate.

In order to examine the factors which influence the lysis of gram-negative bacteria by lysozyme, an alteration in the cell surface by methods other than treatment with heat or acetone was needed. It was observed that cells of Neisseria catarrhalis when pretreated with polymyxin B sulfate (Pfizer) were readily lysed by lysozyme. Since polymyxin is not only bactericidal to gram-negative bacteria but aqueous solutions of the antibiotic are markedly surface active (Newton, 1953) and possess the ability to alter cell permeability (Newton, 1954), it was considered of interest to study the relationship between polymyxin activity and lysis of bacterial cells by lysozyme. Electron microscopic observations were also made on the effect of polymyxin and lysozyme on the cell structure of N. catarrhalis.

MATERIALS AND METHODS

A strain of N. catarrhalis taken from the culture collection of the Wyeth Institute for Medical Research was used in all experiments. The organism was grown in Roux bottles on the surface of a medium consisting (in per cent) of beef extract, 0.3; peptone, 1.0; sodium chloride, 0.5; glycerol, 1.0; and agar, 2.0; final pH 7.6. The bottles were inoculated with 6-hr cultures and incubated at 37 C for 18 to 20 hr. The cells were harvested carefully with a small volume of 0.1 M acetate buffer pH 6.0, and the suspension was standardized to give final turbidity readings of 300 on the Klett-Summerson photoelectric colorimeter with a no. 66 filter.

The cells in 5-ml amounts were distributed in a series of standardized Klett tubes and the suspensions treated with 1 ml of various concentrations of polymyxin B sulfate. The antibiotic-cell suspension was maintained at 25 C for 1 hr, after which the cells were centrifuged in a refrigerated centrifuge (5 C) and suspended in 5 ml of 0.05 M phosphate buffer, pH 7.0. Immediately after this treatment the cells were treated with lysozyme and serial dilutions of the enzyme in 0.1 ml amounts were added to each tube. The tubes were incubated for 30 min in a 37 C water bath and were then read for clearing of the suspensions.

Specimens for electron microscopy were prepared in the following manner: Suspensions of N. catarrhalis in 0.1 M acetate buffer pH 6.0 were centrifuged, washed once with acetate buffer and resuspended in a volume of buffer equal to that of the standardized suspension. The organisms were treated with 20 μg/ml of polymyxin per ml of suspension for 1 hr at 25 C. The cells were centrifuged and then resuspended in 5 ml of 0.05 M phosphate buffer pH 7.0, and 5 μg of lysozyme per ml of suspension was added to the tubes.

Aliquots were removed from the tubes, diluted with phosphate buffer, and mounted on a collodion covered screen. The preparations were shadowed with germanium and viewed in the RCA EMU-3 type electron microscope.

RESULTS

The lysis of polymyxin treated cells by lysozyme. The rates of lysis of treated and untreated suspensions of N. catarrhalis by lysozyme were studied and are presented in figure 1. It is evident that the cells following treatment with polymyxin were readily lysed by the enzyme. The untreated control cells incubated with either lysozyme or polymyxin showed no significant lysis.

1 A crystalline preparation of lysozyme prepared from egg white by Armour Laboratories was used.
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**Figure 1.** Lysis of polymyxin-treated cells of _Neisseria catarrhalis_ by lysozyme. Cone of enzyme: 5 μg/ml. Incubation at 37 C. •—• = cells treated with 20 μg polymyxin/ml for 60 min at pH 6.0 followed by sedimentation of the cells, resuspension in buffer at pH 7.0 and incubation with lysozyme. •—•—• = polymyxin treated cells suspended in buffer alone. • — — — = lysozyme treated cells suspended in buffer alone.

**Figure 2.** Effect of various concentrations of polymyxin upon the lysis of _Neisseria catarrhalis_ by lysozyme. Cone of enzyme: 5 μg/ml. Incubated 30 min at 37 C. •—• = cells treated with polymyxin and lysozyme. •—•—• = cell suspensions incubated with polymyxin alone.

**Effect of polymyxin concentration on the lysis of cells.** Since it was apparent that the lysis of _N. catarrhalis_ by lysozyme was dependent on pretreatment of cells by polymyxin, the effect of various concentrations of the antibiotic on lysis was determined. The course of lysis is shown in figure 2. With increasing concentration of polymyxin the activity of lysozyme was found to increase and concentrations up to 20 μg/ml caused a marked lysis of the cells; further increase in concentration of the antibiotic had little additional effect.

**Effect of lysozyme concentration on the lysis of cells.** Turbidity measurements performed on suspensions of polymyxin treated cells of _N. catarrhalis_ containing several concentrations of lysozyme are illustrated in figure 3. The addition of increasing concentrations of lysozyme from 0.3 to 4.0 μg/ml caused a progressive increase in lysis of the organisms. With concentrations above 4.0 μg/ml the lysis curve was not different from that caused by 8.0 μg/ml. Lysozyme, by itself, had practically no effect on cells of _N. catarrhalis_ which had not been previously treated with the antibiotic.

**Figure 3.** Effect of lysozyme concentration upon the lysis of polymyxin treated cells of _Neisseria catarrhalis_. Cone of polymyxin: 20 μg/ml. • — • = cells treated with polymyxin and lysozyme. •—•—• = cells suspended in lysozyme alone.

**Figure 4.** Effect of temperature of incubation with polymyxin on lysis of _Neisseria catarrhalis_ by lysozyme. Cone of polymyxin: 20 μg/ml; cone of lysozyme: 5 μg/ml.
Effect of temperature of incubation with polymyxin on lysis by lysozyme. Studies were made to determine whether the temperature at which the cells were incubated with polymyxin affected the action of lysozyme. Buffered (pH 6.0) cell suspensions containing 20 µg/ml of polymyxin were maintained for 1 hr at 5°C and 25°C. After the period of exposure, the cells were separated by centrifugation and suspended in 5 ml of .05 M phosphate buffer (pH 7.0) containing 5 µg/ml of lysozyme. The results are shown in figure 4. It is apparent that the cells were lysed by the enzyme at approximately the same rate following incubation with polymyxin at 5°C and 25°C. In addition, no significant alteration in the reaction rate was produced following incubation of cells with polymyxin at 37°C. The fact that no appreciable decrease in rate of lysis occurred at 5°C suggests that polymyxin action was not enzymatic and that the antibiotic-cell action takes place immediately.

Action of lysozyme on polymyxin sensitive gram-
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Figure 6. Normal cells of Neisseria catarrhalis. Magnification, × 11,550.

Figure 7. Neisseria catarrhalis exposed to polymyxin (20 µg/ml) for 60 min. Magnification, × 11,550.

Figure 8. Neisseria catarrhalis exposed to lysozyme (5 µg/ml) for 30 min. Magnification, × 11,550.

Figures 9-11. The appearance of cells of Neisseria catarrhalis after treatment with polymyxin for 60 min at 25°C. Cells centrifuged and sediment resuspended in buffer (pH 7.0) and exposed to 5 µg/ml lysozyme for 30 min. Magnification, × 11,550.

negative bacteria. Since polymyxin is bactericidal to a number of gram-negative bacteria which contain a substrate for lysozyme (Warren et al., 1955), it seemed of interest to include several of these organisms in this study. It appeared likely that treatment of these organisms with polymyxin might result in extensive lysis following the addition of lysozyme. The cultures studied were strains that had been maintained in a laboratory culture collection for several years. The organisms were found to be sensitive to polymyxin in concentrations between 1.0 and 7.8 µg/ml as determined by the broth dilution method of Kagan et al. (1951). Figure 5 presents the results of a typical experiment. Although differences in sensitivity to lysozyme were observed, no correlation could be demonstrated between the sensitivity of the organisms to the antibiotic and the lytic response following the addition of the enzyme. Thus, N. catarrhalis and Pseudomonas aeruginosa possessed similar sensitivities to polymyxin, i.e., 1.0 and 1.4 µg/ml, respectively. However, N. catarrhalis was much more sensitive to lysis by lysozyme and higher concentrations of polymyxin were required to render the cells of P. aeruginosa susceptible to lysozyme. The latter
organism also showed appreciable lysis in the presence of polymyxin alone. Additional organisms, e. g., Salmonella typhi-murium and Phytomonas tumefaciens that were sensitive to polymyxin in concentrations of 1.0 to 1.9 μg/ml were also studied under similar conditions to those used with the other organisms. However, no lysis of the polymyxin treated cells was detected on incubation with lysozyme.

Electron microscopical observations of polymyxin and lysozyme-treated cells. The results of experiments with lysozyme following treatment of the organism with polymyxin are demonstrated in figures 6 to 11. Figure 6 shows control cells of N. catarrhalis suspended in buffer alone, whereas figures 7 and 8 depict the effect of treatment with polymyxin and lysozyme, respectively. The cells treated with either lysozyme or polymyxin showed no morphologic changes when compared with the normal cells. However, considerable damage was evident upon the addition of lysozyme to cells previously treated with polymyxin. The cells (figures 9–11) did not retain their electron dense material and the cell walls showed evidence of disintegration. The presence of a large amount of debris and cell fragments as well as an exudation of protoplasmic constituents were also apparent.

DISCUSSION

The present experiments have shown that pretreatment of cells of N. catarrhalis with polymyxin B sulfate resulted in marked lysis of the cells following the addition of lysozyme. Considerable evidence now exists which would indicate that the activity of polymyxin on susceptible organisms is due to its ability to disorganize structures of the bacterial cell wall which are responsible for the maintenance of the osmotic equilibrium of the cell (Newton, 1956). Since neither polymyxin nor lysozyme acting independently on cells of N. catarrhalis resulted in lysis of the cells, the results achieved with polymyxin and lysozyme would suggest that pretreatment with the antibiotic increased the permeability of the cells or disorganized the cell wall and hence rendered the mucopolysaccharide substrate accessible to the action of the enzyme.

That the effect of polymyxin on cell lysis by lysozyme was not specific for N. catarrhalis is shown by the observations presented in figure 5 wherein polymyxin treatment has increased the susceptibility of several gram-negative bacteria to lysis by lysozyme. However, several organisms which were quite sensitive to polymyxin, e. g., Erwinia carotovora, S. typhi-murium, P. tumefaciens, were found to be either somewhat resistant or completely refractory to lysis by lysozyme. Since the different susceptibilities of the various organisms to lysozyme action cannot be explained in terms of the quantity of the enzyme substrate in the cell wall or to sensitivities to polymyxin, it is highly probable that the extent of physical disorganization of the bacterial cells by polymyxin varied with the species of organism studied, thereby permitting different thresholds of accessibility of the substrate to lysozyme action.

The reasons for the inability of bactericidal concentrations of polymyxin to render only certain species of gram-negative bacteria susceptible to lysozyme action are not known. Salton (1953) has reported several interesting differences in the lipid, protein and carbohydrate components of the cell walls of gram-negative bacteria which could perform at least one function of providing the cell with a mechanically rigid wall. It has also been shown that gram-negative bacteria possess different amounts of high molecular weight polysaccharides (Warren, 1951; Warren and Gray, 1954), and these polysaccharides might act as permeability barriers in some organisms in suppressing lysozyme action. A recent observation by Skarnes and Watson (1955) in which large acidic polymers were found to effectively inhibit lysozyme activity would appear to support the concept that bacterial polysaccharides may neutralize lysozyme. It seems likely, therefore, that resistance of polymyxin sensitive bacteria to lysis by lysozyme is governed by the nature of the cell wall and that a bactericidal concentration of polymyxin may not sufficiently disorganize the cell wall to permit lysis by lysozyme.

SUMMARY

Lysis of Neisseria catarrhalis, as well as other gram-negative bacteria by lysozyme has been demonstrated in cells pretreated with polymyxin B sulfate. Evidence is presented which indicates that polymyxin action is not enzymatic and that antibiotic-cell action takes place immediately at the temperature range studied.

Electron micrographs of polymyxin treated...
cells of *N. catarrhalis* show no alteration in the surface structure of the cells. This observation together with the marked damage of the cells following lysozyme treatment reflects a disorganization of the cell wall by a combination of the polymyxin with the cell.

On the basis of the experiments described it is suggested that polymyxin action results in an increase in permeability of the cells or a disorganization of the cell wall and renders the mucopolysaccharide substrate accessible to the action of lysozyme.

REFERENCES


