Pneumococcal Beta Hemolysin Produced Under the Effect of Antibiotics

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The relationship between exposure of pneumococci to antibiotics and appearance of beta hemolysis (rather than the usual alpha hemolysis) was studied in 100 isolates. All strains were capable of producing beta hemolysis. This occurred at the edge of inhibition zones produced by methicillin and other antibiotics, but only if grown anaerobically and subsequently exposed to air at reduced temperatures. Autolysis of the pneumococci was necessary for the beta hemolysis to be produced. Beta hemolysis was optimal at pH 6.8; none occurred at pH 7.4. The concentration of red cells influenced the reaction: at 4% the extent of beta hemolysis was drastically reduced, which suggests that the lysin is not an enzyme.

A pneumococcal hemolysin was first reported by Libman in 1905 (4). It lysed red cells of sheep, rabbit, horse, and man (2, 3) suspended in liquid medium, and it was formed equally well under aerobic and anaerobic conditions (7). Although pneumococci characteristically produce alpha hemolysis of red cells in agar, it has recently been reported that 39% of the strains of pneumococci produce beta hemolysis on horse blood agar when grown anaerobically and subsequently exposed to air at 6°C (6).

Staphylococci, when exposed to some of the beta-lactam antibiotics, produce beta hemolysin on sheep and rabbit blood agar which is different from the hemolysis produced by staphylococci not in contact with antibiotics (5). An increase of hemolysis production by staphylococci when grown in the presence of penicillin was also reported (8).

This paper will describe some factors which influence the occurrence of rings of beta hemolysis surrounding the zone of inhibition produced when certain antibiotic disks are placed on plates inoculated with pneumococci.

MATERIALS AND METHODS

One hundred strains of Diplococcus pneumoniae (pneumococci) recently isolated from patients were tested for antibiotic susceptibility and for beta hemolysis on 2% horse red cell-Brucella agar with the following antibiotic sensitivity disks (BBL): penicillin G, 10 units; methicillin, 1 μg; oxacillin, 1 μg; cloxacillin, 1 μg; dicloxacillin, 1 μg; nafcillin, 1 μg; ampicillin, 10 μg; erythromycin, 15 μg; chloramphenicol, 30 μg; tetracycline, 30 μg; streptomycin, 10 μg; lincomycin, 2 μg; carbenicillin, 50 μg; novobiocin, 30 μg; cephalothin, 30 μg; gentamicin, 30 μg; nitrofurantoin, 100 μg; cycloserine, 10 μg; vancomycin, 5 μg; and bacitracin, 0.04 unit.

The following media were used: Brewer thioglycolate broth (BBL); Brucella agar (Difco); defibrinated horse blood (BBL); ethanolamine agar (The Rockefeller Institute, New York) filter membrane no. PHWP04700, PH 0.30, 47 mm (Millipore Corp., Bedford, Mass.). (Ethanolamine agar is a synthetic medium on which pneumococci grow with their cell walls insensitive to autolytic enzymes; therefore, on this medium the pneumococci do not lyse spontaneously and do not lyse either under the effect of bile or beta-lactam antibiotics [91].) The inoculum consisted of pneumococci planted in a tube with 10 ml of Brewer thioglycolate broth maintained in a water bath at 37°C for 5 h. All tests were done on plates containing 12 ml of agar.

All plates, except those used in experiment E, were plated with 0.2 ml of inoculum by streaking with a dacron swab. Two sensitivity disks were placed on each plate. All plates were incubated anaerobically in Brewer jars for 24 h and subsequently exposed to air at 6°C for 48 h. They were examined after 6, 24, and 48 h of exposure to air at 6°C.

The extent of beta hemolysis was evaluated by measuring the width of the rings of beta hemolysis surrounding the zone of inhibition produced with the sensitivity disks.

The following additional experiments were done using only 10 strains of pneumococci and methicillin disks.
Experiment A: washed cells plus serum. Horse red cells were washed five times with saline by repeated centrifugation, and 2 ml of the washed red cells was added to 100 ml of Brucella agar. Horse serum in a proportion of 0.1%, 0.2%, 0.5%, 1%, 2%, 3%, and 4% was added to the Brucella agar-horse red cell medium. Brucella agar with 2% horse red cells, but without horse serum, was used as a control.

Experiment B: various concentrations of red cells. Horse blood was centrifuged at 1,000 rev/min for 0.5 h, and sedimented red cells were added to Brucella agar in the following proportions: 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 5%, 6%, 10%, and 12%.

Experiment C: effect of pH. Phosphate buffer solutions were prepared at pH 6.4, 6.8, 7.2, 7.4, and 7.8, and then 25 ml of buffer was added to 75 ml of water in which the Brucella agar was dissolved. A control without buffer was also prepared. Horse red cells were added in a proportion of 2%, and the pH of the media was then checked with a Corning pH meter. The pH of the blood agar without buffer was 6.7.

Experiment D: effect of ethanolamine agar. Ethanolamine agar containing 2% washed horse red cells and control plates with Brucella agar also containing 2% washed horse red cells were inoculated at the same time. After 24 h of anaerobic incubation at 37 C and another 48 h of exposure to air at 6 C, prints on cover glasses were made from the pneumococci grown around the zones of inhibition on both ethanolamine agar and Brucella agar. The prints were Gram-stained, examined, and photographed.

Experiment E: effect of diffusible substance. Millipore filter membranes were placed on plates with Brucella agar-horse red cells, and 0.1 ml of inoculum was spread on the membrane, two plates for each strain. A disk of methicillin was placed in the center of the membrane. The plates were incubated anaerobically for 24 h at 37 C, following which the membranes were removed and discarded. After the membrane was removed, one plate for each strain was then exposed to air at 37 C and the other to air at 6 C, and the plates were examined for beta hemolysis after 48 h.

RESULTS

All 100 pneumococcal strains produced a ring of beta hemolysis surrounding the zones of inhibition produced with two or more antibiotics when incubated anaerobically for 24 h at 37 C and subsequently exposed to air at 6 C for 48 h (Fig. 1). Many strains produced rings of beta hemolysis within the first 24 h of exposure to air at 6 C. All rings of beta hemolysis were preceded by a ring of alpha hemolysis which was observed after 6 h of exposure to air at 6 C. As shown in Table 1, the rings of beta hemolysis were observed mostly around the zone of inhibition produced with some of those antibiotics which inhibit the synthesis of cell wall mucopeptides. Methicillin and oxacillin were associated with the highest incidence as well as with the widest rings of beta hemolysis. Nafcillin, cloxacillin, dicloxacillin, ampicillin, penicillin, carbenicillin, cephalothin, and vancomycin also produced beta hemolysis rings frequently, but they were narrower than those produced with methicillin and oxacillin. There was no relationship between the diameter of the zones of inhibition and the width of the ring of beta hemolysis. No rings of beta hemolysis were observed around the zones of inhibition produced with tetracycline, erythromycin, chloramphenicol, lincomycin, gentamicin, or streptomycin.

The effect of serum (experiment A) is shown in Table 2. The rings of beta hemolysis were narrower on agar with washed red cells (i.e., without serum) than on agar containing 0.1% to 3% serum.

The effect of various concentrations of red cells (experiment B) on the extent of beta hemolysis is presented in Fig. 2. The beta hemolysis was strongest on agar with 2% red cells and slightly less intense with 2.5% and 3%. It dropped considerably at a red cell concentration of 4%, falling almost to zero at 5% and over.

The pH effect (experiment C) is shown in Table 3. The most extensive beta hemolysis was produced at pH 6.8. At pH 7 there was a slight decrease in beta hemolysis; at pH 7.2 the
beta hemolysis was substantially reduced; and at 7.4 there was no beta hemolysis.

In experiment D, 7 of the 10 strains tested produced no hemolysis on ethanolamine-red cell agar and the three other strains produced only a ring of red discoloration around zones of inhibition. All strains produced extensive rings of beta hemolysis on Brucella-horse red cell agar. The Gram-stained prints made on cover glasses from the Brucella agar showed intense autolysis of pneumococci (almost complete disappearance of gram-positive forms), while the prints taken from ethanolamine agar showed swollen gram-positive cocci but no autolysed organisms (Fig. 3).

The plates in experiment E, which were exposed to air at 37 C after removal of the membranes, did not show hemolysis. Plates exposed to air at 6 C did show a ring of beta hemolysis.

**DISCUSSION**

It appears that all pneumococci tested have the potential to generate a beta hemolysin when exposed to certain antibiotics. This is in contrast to antibiotic-related beta hemolysin of *Staphylococcus aureus* (5), which was produced only with β-lactam antibiotics.

Hemolysins can be divided into enzymes and nonenzymes (1). Enzymatic hemolysis varies only slightly as a function of the number of red cells exposed, as 1 molecule of hemolysin is supposed to act on many cells sequentially

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**TABLE 1. Number of strains* producing rings of beta hemolysis on horse blood agar: effect of drugs on the intensity of hemolysis**

| Drug          | Strains producing beta hemolysis | Total strains producing beta hemolysis |
|---------------|----------------------------------|----------------------------------------|
|               | 0-2 mm*                          | 2-5 mm                                 |
| Penicillin    | 24 10 44                           | 76                                     |
| Methicillin   | 0 3 30                              | 100                                    |
| Oxacillin     | 0 2 44                              | 100                                    |
| Cloxacillin   | 1 24 38                             | 99                                     |
| Dicloxacillin | 5 8 65                              | 95                                     |
| Nafcillin     | 5 6 45                              | 95                                     |
| Ampicillin    | 14 3 54                             | 86                                     |
| Carbenicillin | 24 1 57                             | 76                                     |
| Cephalothin   | 29 21 39                            | 71                                     |
| Vancomycin    | 20 16 49                            | 80                                     |
| Cycloserine   | 80 8 10                             | 20                                     |
| Streptomycin  | 71 16 13                            | 29                                     |
| Gentamicin    | 100                                |                                        |
| Tetracycline  | 100                                |                                        |
| Chloramphenicol | 100                               |                                        |
| Erythromycin  | 100                                |                                        |
| Lincomycin    | 100                                |                                        |
| Novobiocin    | 89 10 1                             | 11                                     |
| Gantiracin    | 80 12 7                             | 20                                     |
| Nalidixic acid| 64 3 19                              | 36                                     |
| Nitrofurantoin| 88 12                               | 12                                     |

*One hundred strains of *Diplococcus pneumoniae* were investigated.

*a* Width of hemolysis ring produced.

**TABLE 2. Effect of horse serum on the extent of beta hemolysis**

| Serum conc (%) | 3 | 10 | 14 | 25 | 38 | 42 | 57 | 61 | 62 | 63 |
|----------------|---|----|----|----|----|----|----|----|----|----|
| 0 (washed red cells) | 4 | +  | 0  | ++ | +++| 0  | +  | +  | +  | +  |
| 0.1             | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 0.2             | ++| +++| +++| +++| +++| +++| +++| +++| +++| +++|
| 0.5             | +++| +++| +++| +++| +++| +++| +++| +++| +++| +++|
| 1               | +++| +++| +++| +++| +++| +++| +++| +++| +++| +++|
| 2               | +++| +++| +++| +++| +++| +++| +++| +++| +++| +++|
| 3               | +++| +++| +++| +++| +++| +++| +++| +++| +++| +++|
| 4               | +  | +  | +  | +  | ++ | ++ | ++ | ++ | ++ | ++ |

*a* 0, no hemolysis; +, slight hemolysis 0 to 2 mm width of beta hemolysis; ++, moderate hemolysis 2 to 5 mm width of beta hemolysis; ++++, maximal hemolysis 5 to 10 mm width of beta hemolysis.
Table 3. Effect of pH on the extent of hemolysis

| pH of agar | 3 | 10 | 14 | 25 | 38 | 42 | 57 | 61 | 62 | 63 |
|------------|---|----|----|----|----|----|----|----|----|----|
| 6.4        | ++* | +++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 6.7 (no buffer added) | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 6.8        | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 7.0        | +++ | ++ | +++ | +++ | +  | 0  | +++ | ++ | ++ | 0  |
| 7.2        | ++ | 0  | ++ | +  | 0  | 0  | ++ | 0  | +  | 0  |
| 7.4        | +  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| 7.7        | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |

*0, +, ++, ++++, as in Table 2.

Fig. 3. Gram-stained prints of pneumococci from the edge of the zone of inhibition produced with disks of methicillin. On Brucella agar-horse red cells there is almost complete lysis of pneumococci (left). On ethanolamine agar-horse red cells the pneumococci show abnormal forms, but no lysis (right).

Without being used up, and percentage of hemolysis would tend to remain constant with increasing cell concentration. In contrast, non-enzymatic hemolysins are expected to be consumed in the reaction and thus act only once. Therefore, an increase in the red cell concentration would be expected to produce a sharp fall in percentage of hemolysis produced by the latter mechanism. It appears that the beta-hemolytic substance produced by pneumococci in the presence of methicillin is not an enzyme because its activity dropped sharply when the red cell concentration was increased 3% to 4%. The fact that hemolysis occurs only at low temperature (6°C) also suggests that it is not an enzymic reaction.

No hemolysis was produced on the ethanolamine agar. This indicates that some cell wall autolysis is necessary for this beta-hemolytic substance to be produced or released, or both, into the environment. This could also explain why pneumococci exposed to those antibiotics which cause cell wall damage substantially increase their ability to produce beta hemolysis.

The hemolysis produced on plates subsequent to the removal of the membranes shows that the beta-hemolytic substance was produced and released into the agar through the membrane during the 24-h anaerobic incubation. Hemolysis did not occur in air at 37°C; it did occur at 6°C. This indicates that the
exposure to air at 6 C made possible the beta hemolysis in the absence of the pneumococci.

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