Pneumococci Producing Beta Hemolysis on Agar

VICTOR LORIAN and BODUN POPOOLA

Department of Pathology, Division of Microbiology and Epidemiology, The Bronx-Lebanon Hospital Center, Bronx, New York 10466, and Department of Microbiology, The Mount Sinai School of Medicine, New York, New York 10029

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Fifty-six strains of pneumococci were studied for hemolysis on blood-agar. Twenty-two (39%) of these strains produced beta hemolysis on agar containing horse red cells, six (11%) were beta hemolytic for sheep cells, and none lysed human or rabbit red cells. The substance producing beta hemolysis appeared after 24 hr of anaerobic incubation. Subsequent exposure to air at low temperature (6 to 20 C) for 48 hr was needed to activate it. There was no relation between serological type and beta hemolysis production. This substance appears to be different from the pneumococcal hemolysin previously described.

The pneumococcus is an organism customarily described in textbooks as producing alpha hemolysis on blood-agar (3, 9). In liquid medium, the pneumococcus produces a hemolysin active on red cells of sheep, rabbit, horse, and man (1, 2). This pneumococcal hemolysin is a protein with a molecular weight of approximately 63,000 (4). Morch (6) reported that pneumococci produce a distinct alpha hemolysin on 5% horse blood-agar with "more or less pronounced clearing of the medium"; she did not indicate whether this was beta hemolysis.

This paper describes the conditions under which some strains of pneumococci produce beta hemolysis on agar containing red cells from a variety of species of mammals.

MATERIALS AND METHODS

Fifty-six strains of Streptococcus pneumoniae (pneumococci) were typed and tested for hemolysis shortly after their isolation from patients. The strains were subcultured on horse blood-agar in CO2 every 3 to 4 days, 25 times, and then were retested for hemolysis.

The media used were Brewer thioglycolate broth (BBL) and blood-agar plates containing 12 ml of Brucella agar (Difco) with 2% horse, sheep, rabbit, or human red cells (defibrinated blood (BBL and Scott Laboratories, Fiskeville, R.I.) was centrifuged at 1,000 rev/min for 30 min, the supernatant fluid was discarded, and 2 ml from the sedimented red cells was added to 100 ml of Brucella agar). The typing sera used were pneumococcus typing antisera, types 1 to 45 (Copenhagen Statens Seruminstitut, Copenhagen, Denmark).

Each strain was planted in thioglycolate broth and incubated in a water bath at 37 C for 5 hr. Blood-agar plates (one plate for each kind of red cells) were inoculated with pneumococci (grown in thioglycolate) by streaking with a loop so that isolated colonies would develop. Control plates were incubated at 37 C for 24 hr in 5% CO2 or in air, and duplicate plates were incubated anaerobically in Brewer jars at 37 C for 24 hr. The plates were then kept aerobically at 6 C for 72 hr. The plates were examined after 24 hr of incubation and after 6, 24, 48, and 72 hr of exposure to air at 6 C. The hemolysis was recorded at 48 hr, as follows: 1+ indicated that at least one colony, but no more than about 10% of the colonies, showed a clear transparent zone of beta hemolysis surrounding the colony; 2+ indicated that about half of the colonies, and 3+, that at least 90% of the colonies showed beta hemolysis. All of the strains which produced beta hemolysis were retested, as follows: each strain was planted on horse and sheep blood-agar and incubated anaerobically at 37 C for 24 hr. Subsequently, the plates were maintained for 48 hr anaerobically or aerobically at 37, 22, or 6 C. Also, each strain was grown for 24 hr at 37 C in an atmosphere of 5% CO2, and was subsequently kept for 48 hr aerobically at 37, 22, or 6 C. Degree of hemolysis was recorded.

The colonies from the strains producing 3+ hemolysis were examined microscopically, and photographs were taken with a Leitz photomicroscope (Orthomat).

RESULTS

No beta hemolysis was seen around colonies incubated in air or 5% CO2, whether or not there was subsequent exposure to air at 6 C. However, alpha hemolysis was seen regularly. After 24 hr of anaerobic incubation, growth was abundant; the blood-agar showed a
pinkish color (as it usually does after anaerobic incubation), but no discoloration or hemolysis was observed. After 6 hr of exposure to air at 6 C, the colonies turned greenish, and a zone of alpha hemolysis was visible surrounding the colonies. After 24 hr of exposure to air at 6 C, the picture was similar to that observed at 6 hr, but the alpha hemolysis was more pronounced, and in some instances there was beta hemolysis.

The proportion of strains and the percentage of colonies in each strain producing beta hemolysis on the four species of red cells, after anaerobic incubation and subsequent exposure to air at 6 C for 48 hr, is shown in Table 1. Thirty-nine percent of the strains produced beta hemolysis on horse blood-agar, and 11% of the strains (the same which produced beta hemolysis on horse red cells) produced beta hemolysis on sheep blood-agar. No hemolysis was observed on human and rabbit blood-agar. No strain produced 3+ hemolysis on sheep blood-agar, and one-third of the beta-hemolytic strains (13% of all strains tested) produced 3+ hemolysis on horse blood-agar (Fig. 1).

After 72 hr, some strains which had previously showed no beta hemolysis exhibited a few colonies surrounded with beta hemolysis on both horse and sheep blood-agar.

Table 2 shows the results obtained with the pneumococcal strains which produced beta hemolysis on horse blood-agar when grown and maintained under different conditions. All strains produced beta hemolysis when grown anaerobically and maintained for 48 hr in air at 6 C. Only 18% of the same strains produced beta hemolysis when grown anaerobically but maintained in air at 22 C, and the proportion of colonies producing such beta hemolysis was smaller. No hemolysis occurred when the strains were grown anaerobically and subsequently kept for 48 hr in air at 37 C or anaerobically at 6, 22, or 37 C. There was no beta hemolysis when the strains were grown in CO₂ and subsequently maintained in air for 48 hr at 6, 22, or 37 C.

Microscopic examination of the colonies producing beta hemolysis showed that the thickness of a colony was about 8 to 10 μm. Beneath the colony, there was a layer of 40 to 50 μm showing beta hemolysis, under which the red cells appeared to be intact, but with a greenish coloration. A ring of intact red cells about 80 to 100 μm in width, adjacent to the colony, separated the colony from a zone of beta hemolysis 0.5 to 1.5 mm wide (Fig. 2 and 3). The area of beta hemolysis showed discoloration and lysis of red cells. The pattern of beta hemolysis produced with recently isolated strains was comparable to the pattern obtained with the same strains after they were subcultured 25 times. There was no apparent relationship between certain serological types and

![Horse blood-agar plate with pneumococci showing beta hemolysis.](http://aem.asm.org/)

### Table 1. Production of beta hemolysis by pneumococcal colonies on blood-agar when incubated anaerobically for 24 hr at 37 C and then exposed for 48 hr to air at 6 C

| Species of red cells | No. of strains producing various degrees of hemolysis* | Total no. of strains producing beta hemolysis |
|----------------------|-----------------------------------------------|----------------------------------|
|                      | No hemolysis | + | ++ | +++ |                               |                                |
| Sheep                | 50 (89%)     | 5 (9%) | 1 (2%) | 0 | 6 (11%)                      |                                |
| Horse                | 34 (61%)     | 7 (12%) | 8 (14%) | 7 (13%) | 22 (39%) |                                |
| Man                  | 56           | 0 | 0 | 0 | 0 |
| Rabbit               | 56           | 0 | 0 | 0 | 0 |

*The same 56 strains were tested with red cells of the various species. The degrees of hemolysis were recorded as follows: +, 1 to 10% of the colonies showed beta hemolysis; ++, up to 50% of the colonies showed beta hemolysis; ++++, more than 90% of the colonies showed beta hemolysis.
TABLE 2. Hemolysis on horse blood-agar under various conditionsa

| Strain typeb | Incubated anaerobically at 37 C for 24 hr and then maintained for 48 hr in air atc |
|-------------|---------------------------------------------------------------------------------|
|             | 37 C | 22 C | 6 C |
| 3           | 0    | 0    | +  |
| 23          | 0    | 0    | +  |
| 8           | 0    | 0    | +  |
| 3           | 0    | 0    | +  |
| 9           | 0    | 0    | +++|
| 6           | 0    | 0    | ++ |
| 29          | 0    | 0    | ++ |
| 19          | 0    | 0    | +++|
| 12          | 0    | +    | ++ |
| 23          | 0    | 0    | +++|
| NT1         | 0    | +    | +  |
| NT          | 0    | 0    | ++ |
| 1           | 0    | +    | +++|
| NT          | 0    | 0    | +++|
| 9           | 0    | 0    | +  |
| 11          | 0    | 0    | +  |
| 2           | 0    | 0    | ++ |
| 23          | 0    | 0    | +++|
| 9           | 0    | 0    | +++|
| NT          | 0    | 0    | ++ |

a No strain produced beta hemolysis if incubated for 24 hr at 37 C in CO2, or in air, or if incubated for 24 hr at 37 C anaerobically and subsequently maintained anaerobically at 37, 22, or 6 C for 48 hr.
b The 34 strains which did not produce beta hemolysis were of the following types (superscript refers to the number of strains of indicated type): 12, 34, 4, 5, 6, 8, 9, 11, 12, 17, 18, 19, 21, 23, 29, NT1.
c Zero indicates no hemolysis; +, ++, and +++ are defined in Table 1.
d Not typable.

beta hemolysis production. Of the 56 strains, 22 produced beta hemolysis on all four batches of horse red cells studied. An additional eight strains produced hemolysis on one to three of the four batches.

DISCUSSION

Anaerobic growth and subsequent exposure to air at a temperature of between 6 and 22 C are necessary conditions for the production of beta hemolysis. More than one-third of the pneumococci investigated produced, under these conditions, beta hemolysis on horse blood-agar and, in a much lesser degree, on sheep blood-agar. No strain produced hemolysis on rabbit or human blood-agar.

Peroxides which are formed under aerobic growth of streptococci are one of the causes of the greenish coloration of the colonies (alpha hemolysis), and affect the red cells in such a way as to prevent their further hemolysis (8). Although the green alpha hemolysis produced by pneumococci is generated by the same substances (5), the subsequent beta hemolysis of the greenish-red cells was not prevented. In fact, the greenish alpha hemolysis preceded closely the clearing which led to beta hemolysis.

The hemolysin (pneumolysin) produced by pneumococci in liquid media, as known here-tofore (1, 2), is generated under both aerobic and anaerobic incubation, is active on four types (human, sheep, rabbit, horse) of red cells (2) at 37 C, and is inactivated by oxidation (7). The substance producing beta hemolysis on blood-agar described in this paper appears only after anaerobic incubation, does not hemolyze human and rabbit red cells, and needs air and low temperatures to be activated. It appears, therefore, that this substance is different from the pneumococcal hemolysin previously described (1, 2, 4).

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Fig. 3. Appearance of pneumococcal colonies and medium showing beta hemolysis. (a) Two colonies surrounded by a darker ring (intact red cells) and a clear zone of beta hemolysis. × 40. (b) Surface and edge of a colony. A ring of red cells stands between the colony and the zone of beta hemolysis. × 100. (c) Same focus, but 100 μm deeper. Intact red cells beneath the colony, but beta hemolysis is surrounding the colony.