Effect of Antibiotics on Staphylococcal Hemolysin Production

VICTOR LORIAN

Division of Microbiology and Epidemiology, Department of Pathology, The Bronx-Lebanon Hospital Center, Bronx, New York 10456, and the Department of Microbiology, The Mount Sinai School of Medicine, New York, New York 10029

Received for publication 8 April 1971

Thirty-seven per cent of 126 strains of Staphylococcus aureus, when tested on sheep blood-agar with sensitivity discs containing cephalothin, carbenicillin, oxacillin, penicillin, and cycloserine, produced rings of \( \beta \)-hemolysis surrounding zones of inhibition of bacterial growth. Each strain capable of producing a ring of \( \beta \)-hemolysis did so with at least two of the mentioned antibiotics. None of the other 15 antibiotics tested was associated with a ring of \( \beta \)-hemolysis surrounding any of the zones of inhibition. It appears, therefore, that the \( \beta \)-hemolysis observed was produced by certain strains of S. aureus only in association with certain antibiotics.

\textit{Staphylococcus aureus} can produce at least four hemolysins, alpha, beta, gamma, and delta (4). The most common one is the \( \alpha \)-hemolysin which is most active on rabbit red cells and less active on sheep and human red cells (3). Most of the strains of \textit{S. aureus}, when planted on sheep blood-agar in appropriate inoculum to produce almost confluent growth after 24 hr at 37 C, will produce a slight hemolysis. Very few strains will produce no change at all or, on the contrary, will produce clear-cut \( \beta \)-hemolysis. In examining routine antibiotic sensitivity tests, it was noted that many strains of staphylococci, some strains of \textit{Escherichia coli}, and some strains of \textit{Clostridium perfringens} produce a ring of \( \beta \)-hemolysis surrounding the inhibition zone produced with discs of cephalothin, penicillin G, and semisynthetic penicillins.

The purpose of this paper is to describe some factors influencing the occurrence of \( \beta \)-hemolysis surrounding the zones of inhibition produced when antibiotic discs are placed on plates inoculated with \textit{S. aureus}.

MATERIALS AND METHODS

A total of 126 strains of \textit{S. aureus} recently isolated from patients were tested for hemolysis production. All were sensitive to penicillin, oxacillin, ampicillin, carbenicillin, and cephalothin and produced no distinct \( \beta \)-hemolysis on sheep blood-agar when planted so that almost confluent growth occurred. Tests for hemolysis and for antibiotic susceptibility were performed on plates containing 16 ml of Trypticase Soy agar (Difco) with 5% sheep red cells, 5% washed human group O red cells, 5% horse red cells, or 5% rabbit red cells, all prepared not more than 5 days before use (Scott Co., Fiskeville, R.I.). The following antibiotic sensitivity discs (BBL) were used: penicillin G, 10 units; ampicillin, 10 \( \mu \)g; oxacillin, 1 \( \mu \)g; erythromycin, 15 \( \mu \)g; chloramphenicol, 30 \( \mu \)g; tetracycline, 30 \( \mu \)g; streptomycin, 10 \( \mu \)g; kanamycin, 30 \( \mu \)g; neomycin, 30 \( \mu \)g; lincomycin, 2 \( \mu \)g; carbenicillin, 50 \( \mu \)g; novobiocin, 30 \( \mu \)g; cephalothin, 30 \( \mu \)g; polymyxin B, 300 units; nitrofurantoin, 100 \( \mu \)g; nalidixic acid, 30 \( \mu \)g; gentamicin, 10 \( \mu \)g; cycloserine, 10 \( \mu \)g; vancomycin, 5 \( \mu \)g; and bacitracin, 0.04 units. Plates were inoculated by flooding with a 1:2,000 dilution in sterile saline of a 24-hr Trypticase Soy broth (BBL) culture which contained from 10\(^8\) to 3 \( \times 10^8\) organisms per ml. The reading and interpretation of the results were according to the tables of Bauer et al. (1).

The inhibition zones were examined after 24 hr of incubation at 37 C, and the microorganisms were removed from the plates by washing with saline (so that the hemolysis could be observed more clearly). A ring of \( \beta \)-hemolysis 1 mm in width surrounding the inhibition zone was scored 1+, a ring of 2 mm was scored 2+, and a ring of 3 mm or larger was scored 3+. Absence of \( \beta \)-hemolysis was recorded as zero.

Ten strains that produced 3+ hemolysis rings on sheep blood-agar were tested for “hot-cold hemolysis”; after incubation at 37 C for 24 hr, the plates were kept overnight at 6 C. These 10 strains were also tested anaerobically in Brewer Jars at 37 C for 24 hr for the effect of antibiotic discs on hemolysis.

The effect on hemolysis of a circular area devoid of bacterial growth in the midst of a bacterial lawn was determined by shielding a portion of the agar surface from bacteria at the time of inoculation, as previously described (5). This simulated an anti-
biotic zone of inhibition without exposing either the bacteria or the red blood cells to antibiotic.

RESULTS

Forty-seven (37%) of the 126 strains tested produced a ring of β-hemolysis on sheep blood-agar in association with two or more antibiotics, and 79 (63%) did not produce β-hemolysis with any antibiotic (Table 1).

As shown in Table 2, the rings of β-hemolysis were observed only around the zones of inhibition produced with some antibiotics that inhibit the synthesis of cell wall mucopeptides. In a few instances, narrow rings of β-hemolysis surrounding zones of inhibition were produced with discs of nitrofurantoin, novobiocin, neomycin, and chloramphenicol, but these observations were not reproducible.

| Table 1. Number of strains producing rings of β-hemolysis on sheep blood-agar according to the number of drugs that produced them |
|-----------------------------------------------|
| Determination                             | No. of strains |
|-----------------------------------------------|
| Total no. of strains tested                | 126            |
| Hemolysis with no drugs on plate.           | 0 (0)*         |
| No hemolysis with all drugs.                | 79 (63)        |
| Hemolysis with one drug only                | 0 (0)          |
| Hemolysis with two drugs only               | 11 (9)         |
| Hemolysis with three drugs only             | 14 (11)        |
| Hemolysis with four drugs only              | 10 (8)         |
| Hemolysis with five drugs only              | 8 (6)          |
| Hemolysis with six drugs only               | 4 (3)          |
| Total no. of strains producing a ring of β-hemolysis | 47 (37) |

* Numbers in parentheses represent percentages.

Cephalothin and carbenicillin produced the highest incidence (34%) as well as the widest rings of β-hemolysis, the majority being in the group of 3+ or more (Fig. 1). Oxacillin also produced many β-hemolysis rings (30%), but the majority were in the category of 2+. In only a few instances were the inhibition zones produced with penicillin G, ampicillin, and cycloserine surrounded with rings of β-hemolysis. Vancomycin and bacitracin produced zones of inhibition in all strains tested, but none was surrounded by a ring of β-hemolysis. No ring of β-hemolysis was observed around the zones of inhibition produced with kanamycin (Fig. 1) or the other antibacterial agents tested.

In many instances, an increased bacterial density was noted at the edge of the zones of inhibition around discs of vancomycin, tetracycline, and neomycin, but no ring of β-hemolysis was visible around any of these zones. None of the areas free from growth produced by shielding them from inoculation showed a corresponding ring of β-hemolysis.

All strains produced a complete β-hemolysis throughout agar plates containing rabbit red cells; therefore, rings of β-hemolysis around zones of inhibition were not seen. No strain produced hemolysis around antibiotic discs on agar plates with human red cells or horse red cells. In a very few instances, narrow rings of β-hemolysis were observed on agar plates with human or horse red cells surrounding the zones of inhibition produced with cephalothin, but this observation was not reproducible. Staphylococcal hemolysis of red cells from various sources, with or without antibiotics, is summarized in Table 3. The rings of β-hemolysis on plates kept at 6°C overnight did not show any change. The plates incubated anaerobically showed variable results; some rings of β-hemolysis were wider, most were similar, and some were narrower than those produced under aerobic conditions.

DISCUSSION

The rings of β-hemolysis surrounding the zones of inhibition on sheep blood-agar were observed with 37% of the strains of staphylococci tested and appeared only with certain antibiotics that are mucopeptide synthesis inhibitors and not with bacitracin and vancomycin which also inhibit mucopeptide synthesis. Inhibition zones produced with other antibiotics were not surrounded with β-hemolysis. No ring of β-hemolysis was seen when dense growth at the edge of the simulated zone of inhibition was produced.
Fig. 1. (Top) Blood-agar plate with Staphylococcus aureus strain 7A showing two inhibition zones, a small one produced with a disc of kanamycin and a larger one produced with a disc of carbenicillin. (Bottom) The same plate, illuminated from the back, showing only one ring of β-hemolysis surrounding the inhibition zone produced with the disc containing carbenicillin.

by shielding an area of the plate from the inoculum. Therefore, the appearance of these rings of β-hemolysis was clearly not due to a critical density of organisms near an area without bacterial growth.

The confluent β-hemolysis noted on 5% rabbit blood-agar was most likely due to α-hemolysin which is known to be very active against such cells and needs oxygen and CO₂ to be produced (4). Its production and activity were independent of antibiotic effect, since they occurred on plates without antibiotic discs. In contrast, the rings of
Table 3. Staphylococcal hemolysis of red blood cells from various sources

| Source of red blood cells | No. tested | No. showing hemolysis | Type of hemolysis                          |
|--------------------------|------------|----------------------|-------------------------------------------|
| Sheep                    | 126        | 47                   | \(\beta\), Only associated with certain antibiotics |
| Rabbit                   | 126        | 126                  | \(\beta\), Unrelated to antibiotics        |
| Human                    | 126        | 0                    | No hemolysis                               |
| Horse                    | 126        | 0                    | No hemolysis                               |

\(\beta\)-hemolysis noted on 5% sheep blood-agar were clearly related to the interaction of certain antibiotics and some strains of \(S.\) \textit{aureus} or their hemolysins, or both. They were probably due to a different substance than the \(\alpha\)-hemolysin causing confluent \(\beta\)-hemolysis on rabbit blood-agar because this substance was produced in anaerobiosis (4). It is not a \(\beta\)-lysin because the refrigeration did not intensify it. It could be a \(\gamma\)-lysin because it did not lyse horse red cells, but it is probably not a \(\delta\)-lysin because it is not active on washed human or horse red cells. It is a fact that this hemolysin does not lyse human red cells, but this does not preclude other pathogenic effects in humans, as is the case with \(\alpha\)-lysin (2).

Acknowledgments
This research was supported by Public Health Service grant SO1-FR-055-97-04 from the Division of Research Facilities and Resources.

The author thanks Leon D. Sabath for his assistance in the writing of this paper. Bodun Popoola was the technical assistant in this investigation.

Literature Cited
1. Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. Amer. J. Clin. Pathol. 45:493–496.
2. Bernheimer, A. W., and L. L. Schwartz. 1963. Isolation and composition of staphylococcal \(\alpha\) toxin. J. Gen. Microbiol. 30:455–468.
3. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood. 1967. Microbiology, p. 730. Harper and Row, New York.
4. Dubos, R. J., and J. G. Hirsch, 1965. Bacterial and mycotic infections of man, p. 418. J. B. Lippincott Co., Philadelphia.
5. Lorian, V., and L. Strauss. 1966. Increased bacterial density at the edge of antibiotic zones of inhibition. J. Bacteriol. 92: 1256–1257.