Effect of Fatty Acids on Staphylococcus aureus Delta-Toxin Hemolytic Activity

FRANK A. KAPRAL

Department of Medical Microbiology, The Ohio State University, Columbus, Ohio 43210

Received for publication 22 August 1975

The lysis of human erythrocytes by Staphylococcus aureus delta-toxin proceeded without a lag and was directly proportional to toxin concentration and temperature of incubation. Lysis was complete within 8 min. Addition of saturated, straight-chain fatty acids of 13 to 19 carbons increased the activity of delta-toxin, whereas those with 21 to 23 carbons were inhibitory. Palmitic acid was the fatty acid most active in augmenting delta-toxin, but its effect could be abolished by the simultaneous addition of either tricosanoic acid or egg lecithin.

Previous studies had indicated that certain phospholipids could inhibit the hemolytic activity of Staphylococcus aureus delta-toxin (8). While investigating this phenomenon, a commercial sample of lecithin was obtained which did not inhibit the hemolytic activity of delta-toxin, but, to the contrary, increased its capacity to lyse erythrocytes. The reason for this aberrant behavior was eventually traced to the presence of free palmitic acid in the phospholipid sample. Subsequent studies revealed that delta-toxin activity could be either increased or decreased by the addition of appropriate saturated fatty acids, and which effect was obtained depended upon the length of the fatty acid added. The stimulatory effect produced by palmitic acid could be competitively antagonized by either tricosanoic acid or lecithin. The data suggested that these lipids exerted their effects by influencing the binding of delta-toxin to the erythrocyte.

MATERIALS AND METHODS

Hemolysin. Delta-toxin was prepared and purified as previously described (9). In these studies only the soluble form of delta-toxin was used.

Lipids. Fatty acids were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and Applied Science Labs, Inc., State College, Pa. Egg lecithin was purified as described (8) and stored at −70 C until needed.

Lipids were dissolved in absolute ethanol and stored at −70 C, and such stock solutions were used within 3 days. Working stock suspensions were prepared by rapidly diluting alcoholic stock solutions in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, pH 7.4, with 0.85% NaCl) containing 2.5 mg of gelatin per ml. These suspensions were made immediately before use and discarded after 3 h. Original lipid concentrations were chosen such that, after dilution of alcoholic stocks, the final ethanol concentration in the hemolytic system (see below) did not exceed 2%.

Hemolytic system and kinetics of lysis. Erythrocytes from freshly drawn human blood were washed twice with PBS and resuspended in the same diluent at a 0.4% (vol/vol) concentration. To 25 ml of erythrocyte suspension was added 20 ml of lipid suspension containing the desired amount of fatty acid or lecithin. The mixture was equilibrated at 37 C (or as otherwise noted) in a shaker water bath, and 5 ml of prewarmed delta-toxin in PBS was added. After various intervals, 3-ml aliquots were removed and added to 0.5 ml of lecithin suspension (100 µg) to halt further hemolysis (8). The samples were centrifuged, and the supernatant fluid was assayed for either hemoglobin or potassium. Hemoglobin was determined spectrophotometrically at 541 µm and potassium was measured with a Unicam atomic absorption spectrophotometer operated in the flame photometer mode. The amount of hemoglobin of potassium present in a sample was compared to that released from an equal quantity of erythrocyte suspension lysed by excess delta-toxin. Mixtures consisting of all reactants except delta-toxin served as controls. In experiments in which it was desired only to compare the maximum amount of lysis occurring after a fixed interval of incubation, the volume of the reaction mixtures was reduced, but the proportion of reactants was kept the same.

Pretreatment of erythrocytes with lipids. After washing twice with PBS, 0.25 ml of packed human erythrocytes was resuspended in 25 ml of the same diluent. Either egg lecithin or palmitic acid suspensions (in PBS with gelatin) was added to produce final concentrations of 10 or 4 µg/ml, respectively. After 3 min, the suspensions were centrifuged, and the sedimented cells were washed once in PBS and then resuspended in the same diluent for use in the hemolytic system. Cells pretreated with both lipids were processed in the same manner, except exposure to the first lipid was followed by a single washing in PBS before treatment with the next lipid.
RESULTS

Addition of delta-toxin to the cell suspension resulted in a rapid lysis of erythrocytes without a discernible lag period. Lysis was essentially complete within 8 min, and the extent of lysis at this time was proportional to the amount of delta-toxin added (Fig. 1). The addition of 1 μg of palmitic acid per ml to the system caused a noticeable increase in the lytic activity of toxin (Fig. 2). Palmitic acid, at concentrations used in these studies, caused no lysis in the absence of toxin.

During lysis, potassium release paralleled hemoglobin release, but on a percentage basis more potassium than hemoglobin was discharged (Fig. 3). For a fixed concentration of toxin, increasing amounts of palmitic acid caused greater degrees of lysis (Fig. 4).

The extent of lysis caused by a particular concentration of delta-toxin in the presence or absence of palmitic acid was proportional to the incubation temperature over the range of 5 to 50 C (Fig. 5). For this reason, care was taken to bring all components of the system to the desired temperature before initiating a hemolytic reaction.

A series of saturated, straight-chain fatty acids, varying in chain length, was compared

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![Fig. 1](http://iai.asm.org/) Kinetics of erythrocyte lysis by *S. aureus* delta-toxin at 37 C. Numbers to the right of each curve refer to micrograms of toxin used per milliliter.

![Fig. 2](http://iai.asm.org/) Kinetics of erythrocyte lysis by *S. aureus* delta-toxin at 37 C in the presence of 1 μg of palmitic acid per ml. Numbers to the right of each curve refer to micrograms of toxin used per milliliter.

![Fig. 3](http://iai.asm.org/) Kinetics of hemoglobin and potassium release from erythrocytes lysed at 37 C with 2 μg of *S. aureus* delta-toxin per ml in the presence of 2 μg of palmitic acid per ml.

![Fig. 4](http://iai.asm.org/) Lysis of erythrocytes at 37 C with 2 μg of *S. aureus* delta-toxin per ml in the presence of various concentrations of palmitic acid. Arrows refer to the percentage of hemoglobin or potassium released in the absence of the fatty acid.
for their ability to alter delta-toxin activity (Fig. 6). Fatty acids with chain lengths of 2 to 12 carbons had no effect on toxin activity. Acids of 13 to 19 carbons increased the lytic activity of toxin, with palmitic acid (C16) being the most potent. On the other hand, C21 to C23 fatty acids reduced the activity of delta-toxin.

In addition to inhibiting the lytic activity of delta-toxin, egg lecithin reduced the activity of delta-toxin-palmitic acid mixtures (Fig. 7). The extent of hemolysis resulting from a fixed quantity of toxin could be varied depending upon the relative amounts of palmitic acid and lecithin added to the system (Fig. 8).

Tricosanoic acid (C23), in increasing concentrations, resulted in progressively less hemolysis when mixed with a constant amount of toxin (Fig. 9). The degree of lysis produced with a fixed amount of toxin could be either increased or decreased with the addition of palmitic acid-tricosanoic acid mixtures of various proportions (Fig. 10).

Erythrocytes pretreated with palmitic acid were found to be more susceptible to delta-toxin than cells similarly processed in the absence of the fatty acid (Fig. 11). Such palmitic acid-

treated cells, subsequently treated with lecithin, were restored to about the same degree of sensitivity to toxin as were cells not exposed to palmitic acid. Cells pretreated only with lecithin did not differ significantly from control cells in susceptibility to delta-toxin.

**DISCUSSION**

The rapidity of hemolysis initiated by delta-toxin was first described by Jackson and Little (6). They noted that for a particular toxin concentration hemolysis was essentially maximal in samples first taken 5 min after starting the reaction. We have confirmed these observations but, in addition, have examined more closely the rate of lysis during the first few minutes. This was made possible with the finding that lecithin, when added to the hemolytic
Because of the rapidity of lysis, comparisons of the amount of hemolysis occurring under test conditions could be made after an 8-min incubation period. In almost all instances, additional samples were examined after 30 to 60 min of incubation, but no significant changes were noted after the initial 8-min observation period. Hemolysis could be monitored by measuring either hemoglobin release or potassium release, but supernatant fluids consistently contained a greater percentage of the available potassium than of available hemoglobin. The reason for this preferential release is not clear, but similar observations have been reported with other hemolysins (1).

Although some day-to-day variation in quantitative responses were noted, cells from different blood donors did not significantly affect the results. In initial experiments where lipids were added to reaction mixtures, the amount of hemolysis occurring on different occasions with the same quantity of reactants would vary. This source of variation was, for the most part, eliminated by adding gelatin to the diluent used to prepare lipid suspensions from alcoholic stock solutions. The finding that hemolysis was markedly influenced by the temperature of incubation (Fig. 5) required that, for consistent results, all reactants be equilibrated at the desired temperature before initiating the reaction.

Wiseman and Caird (15) have suggested that delta-toxin is an enzyme with phospholipase C activity and a preference for phosphatidylinositol, but others have failed to substantiate this claim (8, 10, 14). The onset of lysis without an obvious lag, the rapid completion of lysis, system, promptly halted further activity of delta-toxin (8). Therefore, samples could be removed from the reaction mixture at brief intervals and mixed with lecithin and the unlysed cells could be sedimented without concern that lysis would continue unabated during centrifugation. Our resulting data indicated that lysis began without a discernible lag, but, if a lag period existed, its duration was less than 30 s (Fig. 1). The observation by Jackson and Little that the extent of hemolysis was linearly related to the delta-toxin concentration was also confirmed by our studies.

FIG. 9. Lysis of erythrocytes produced at 37 C by 4 μg of S. aureus delta-toxin per ml in the presence of various concentrations of tricosanoic acid. Arrows refer to the percentage of hemoglobin or potassium released by toxin alone.

FIG. 10. Lysis of erythrocytes produced at 37 C by 2 μg of S. aureus delta-toxin per ml in the presence of palmitic acid (C₁₆) and tricosanoic acid (C₂₃) mixed together in various proportions. Horizontal lines indicate the percentage of hemoglobin or potassium released by toxin alone.

Fig. 11. Kinetics of erythrocyte lysis at 37 C by 2 μg of S. aureus delta-toxin per ml, using erythrocytes either pretreated with palmitic acid (and washed) or erythrocytes pretreated with palmitic acid and subsequently treated with purified egg lecithin (and washed). Control erythrocytes were similarly washed, but were not exposed to lipids.
the lack of a temperature optimum, and the apparent stoichiometry of the reaction are findings that suggest that erythrocyte lysis by delta-toxin is not enzymatic in nature.

The mechanism whereby fatty acids influence delta-toxin activity is not clear. The data are compatible with the concept that fatty acids are involved with binding of toxin to the membrane. Fatty acids with chain lengths in the range of palmitic acid could be optimal for binding, whereas those with 21 or more carbons might be too long and interfere. Fatty acids with 12 or less carbons might simply be too short to augment or inhibit attachment. If certain fatty acids are effective coupling agents between the toxin molecule and membrane receptors, the inhibitory effects of phospholipids (8) or Tween 80 (O'Brien and Kapral, unpublished observations) might result from the interaction of these substances with the fatty acids involved in binding. The antagonism observed between palmitic acid and lecithin (Fig. 7 and 8) or between palmitic acid and tricosanoic acid (Fig. 10) suggests a competitive interaction among these lipids which, if true, would lend credence to the hypothesis.

Although fatty acids were compared on an equimolar basis, it must be realized that the concentrations stated in this study are somewhat theoretical, since these lipids were used as dispersions. Efforts were made for consistency in preparation and use of lipid suspensions, but the possibility that the different behaviors associated with various fatty acids might reflect differences in dispersal cannot be rejected.

Free fatty acids alone are capable of lysing erythrocytes, but in the concentrations we used in our study this was not a factor for concern. Controls consisting of erythrocytes and lipid suspensions (at the highest concentrations used) were included with every experiment and revealed no lysis even after 1 h of incubation.

Cells exposed to palmitic acid and then separated from excess fatty acid were more susceptible to delta-toxin than cells similarly washed but not exposed to the lipid (Fig. 11). This increased susceptibility did not appear to result from a permanent alteration of the erythrocyte, since the usual sensitivity could be restored by a subsequent treatment with lecithin. Moreover, these findings could also be construed to support the idea that fatty acids are involved in binding toxin to the membrane.

The question of the antigenicity of delta-toxin has been raised on numerous occasions. The ability of normal sera to neutralize the hemolytic activity of delta-toxin has been observed frequently (2, 6, 10–12), and, although this may be viewed to reflect the presence of antibody, we have suggested that the neutralizing activity results from phospholipids normally present in the serum (8). Attempts to produce antibody to delta-toxin have, for the most part, been reported unsuccessful (4, 5, 7, 13). Recently, Fackrell and Wiseman (3) have claimed production of anti-delta-toxin antibody, based upon their ability to demonstrate formation of a precipitate upon reacting delta-toxin with an immunoglobulin fraction from serum of immunized rabbits and their observation that this serum fraction neutralized the toxin’s hemolytic activity. Although an antibody may have been produced, their findings do not prove the antigenicity of delta-toxin. The precipitation of delta-toxin with sera from unimmunized animals has been noted (7), but this reaction appears to result from combination with some substance other than a globulin. Fackrell and Wiseman did not actually show that the precipitate consisted of delta-toxin and antibody, nor did their data preclude the possibility that the immunoglobulin fraction was contaminated with phospholipids.

Our findings suggest an alternate means of ascertaining whether the neutralizing activity of a serum specimen results from the presence of neutralizing antibody to delta-toxin or solely from the presence of phospholipids. The addition of palmitic acid to a sample should negate the neutralizing activity due to phospholipids, but should not affect the interaction of antigen with antibody. We have found that addition of small amounts of palmitic acid to sera from unimmunized humans or rabbits does indeed abrogate the neutralizing activity present in such specimens, but we have not investigated the effect of fatty acids on immune complexes. Should the latter effect prove consequential, then this approach may be useful in obtaining an answer to a perplexing problem.

ACKNOWLEDGMENT

This study was supported by Public Health Service grant AI-7826 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED