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Staphylococcal Alpha-Hemolysin: Detection on the Erythrocyte Membrane by Immunofluorescence

Abstract: Purified staphylococcal α -hemolysin (but not the toxoid) was demonstrated on the surface of rabbit and human erythrocytes by immunofluorescence. This occurred during the period of maximal hemolysis and was a transient event. These findings have been analyzed in relation to previous data on the kinetics of leakage of both small and complex molecular constituents of the erythrocyte.

While the kinetics of the lytic action of staphylococcal α -hemolysin on cells with rapid leakage of large molecules implicated the cell membrane rather than intermediary metabolism as the primary site of action (1), this point has not hitherto been demonstrated by direct evidence.

Analysis of the time course of hemolysis by highly purified toxin (2) revealed a sigmoid curve with a lag phase before the onset of hemolysis (prelytic lag phase), followed by a period of rapid, linear release of hemoglobin. Release of cell potassium takes place early in the prelytic period, with 50 to 70 percent of this loss occurring before leakage of hemoglobin is detectable (3). Specific antitoxin prevents hemolysis only when added before the end of the prelytic phase (4).

There is disagreement as to whether adsorption of α -hemolysin to the red cell is reversible. Forssman (5) has suggested that crude hemolysin is fixed for a brief time and then released to act on other red cells. On the other hand, Levine (6) has contended that this adsorption is not reversible. Evidence was presented recently by Lominski and Arbuthnott (7) that α -lysin is completely recoverable. The observa-

tions of Cooper *et al.* (4) indicate that, when purified hemolysin has reacted with erythrocytes, the products of hemolysis inhibit its attachment to other cells.

The initial reaction between staphylococcal α -hemolysin and the erythrocyte is probably a surface phenomenon. We have used the fluorescent antibody technique (8, 9) to study this and to attempt to relate the findings to the kinetics of hemolysis of rabbit and human erythrocytes. Purified α -hemolysin [40,000 hemolytic units (HU) per milliliter] was prepared by the method of Madoff and Weinstein (10). This material, when treated by incubation with 0.2 percent formalin (final concentration) at 37°C for 20 hours, was no longer hemolytic. It formed a precipitate with staphylococcal antitoxin (11) and induced active immunity in mice. A γ -globulin fraction of rabbit antibody to horse γ -globulin, conjugated with fluorescein isothiocyanate (9, 12, 13), was mixed in a ratio of 7 parts to 3 parts of normal human serum containing 500 mg per 100 ml of Evans blue dye for counter staining. Fresh erythrocytes were collected daily into Alsever's solution, washed, and prepared as a 2 percent suspension in phosphate-buffered saline, pH 6.9. Hemoglobin and potassium release were determined by methods described previously (2, 3).

The erythrocytes were mixed with purified hemolysin and the suspension was incubated in a water bath at 37°C, the test tubes being subjected to constant agitation. At intervals, excess antitoxin was added directly to samples of the cell-hemolysin suspension and incubated at 37°C for 15 minutes. The cells were centrifuged and washed twice with phosphate-buffered saline. The fluorescein-labeled antibody to horse γ -globulin was added and the mixture was incubated at 37°C for 30 minutes and centrifuged; the cells were washed three times with the phosphate buffer. The cell buttons were then smeared on glass slides, air dried, mounted with a solution containing 9 parts glycerol to 1 part veronal buffer (0.01M, pH 8.6), and examined by fluorescent microscopy.

In a simultaneous study, red cells exposed to hemolysin and antitoxin, as described above, were immediately smeared, air dried, fixed in acetone at -70°C for 2 hours, washed with phosphate-buffered saline, and covered with the fluorescein conjugate in moist

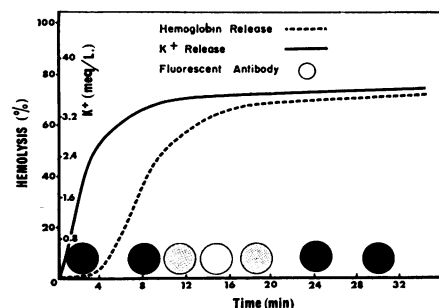


Fig. 1. Time course of hemolysis and release of potassium ions correlated with immunofluorescence when rabbit erythrocytes are exposed to 25 hemolytic units per milliliter of purified staphylococcal α -hemolysin. The gradient of brilliance of fluorescence is designated by the shading of the red cells (indicated by circles along the abscissa): black indicates no fluorescence; stippled, moderate fluorescence; white, bright fluorescence.

petri dishes at 37°C for 1 hour. The preparations were then washed twice with the phosphate buffer, once with veronal buffer, and mounted. Standard controls to document specific fluorescence (9) were included in each study.

More cells became fluorescent when conjugate was added directly to erythrocytes in a test tube and the mixture agitated for 30 minutes than when a red cell smear was flooded with conjugate. This was not unexpected, since there is a greater opportunity for contact between conjugate and erythrocytes with the former method. In each case, however, many cells remained unstained; this reflects the natural



Fig. 2. Rabbit erythrocytes treated with 45 hemolytic units of purified staphylococcal α -hemolysin (45 HU per milliliter of cell suspension) for 15 minutes and then exposed to anti-hemolysin, washed, and stained with fluorescent antibody on slide ($\times 120$). The large cells with the granular cytoplasm and nonfluorescent nuclei are leukocytes.

failure of a portion of an erythrocyte population to hemolyse when exposed to α -hemolysin (14).

The usual differences between rabbit and human erythrocytes were confirmed. The course of hemolysis of rabbit erythrocytes exposed to 25 to 65 HU of purified α -hemolysin per milliliter of cell suspension was identical to that described previously (2-4), the release of potassium ions in the prelytic lag phase being followed by a period of rapid linear release of hemoglobin (Fig. 1). Specific fluorescence (9), manifested by erythrocytes and red cell debris showing fluorescence of variable intensity (Fig. 2), was first demonstrated near the peak of the hemolytic curve, became most intense at the time of maximum hemolysis, and gradually lessened in brilliance and disappeared (Fig. 1). Fluorescence was no longer demonstrable at 25 and 120 minutes when the cells were exposed to 25 and 65 HU, respectively. Exposure to at least 15 HU of α -hemolysin was necessary before the phenomenon could be demonstrated. Specific fluorescence was never noted in control suspensions.

Fluorescence was not demonstrated after human erythrocytes had been exposed to 45 or 65 HU of α -hemolysin for periods as long as 4 hours. When incubated with 5000 HU, fluorescein-labeled cells could be detected after 5 minutes; the fluorescence was most intense after 60 to 120 minutes and then gradually diminished. Minimal fluorescence was still visible at 4 hours. It was evident that, when fluorescence could be seen, hemolysis was also occurring. Fluorescence could not be demonstrated in either rabbit or human erythrocytes exposed to α -hemolysin toxoid.

Our inability to demonstrate the adsorption of toxoid to rabbit red cells suggests that the addition of formalin to staphylococcal α -hemolysin may change the structure of the toxin molecules so that, despite the similarity to unaltered toxin evident in its ability to precipitate the specific antitoxin and to induce active immunity, the α -hemolysin can no longer attach to the erythrocyte surface.

The failure of human erythrocytes to fluoresce with small quantities of α -hemolysin and the high degree of fluorescence observed with very large doses are compatible with the natural resistance of these cells to purified staphylococcal α -hemolysin (15). This

may reflect the relative unavailability of appropriate receptor sites or the necessity for interaction with a large number of toxin molecules before damage occurs. The latter is improbable since, if it were true, it should have been possible to demonstrate fluorescence during the early stages of hemolysis. The data indicate that adsorption of the toxin to the surface of the human erythrocyte is transient and that detachment gradually takes place.

The specific fluorescence observed in these studies must be interpreted as a visual demonstration of the interaction of staphylococcal α -hemolysin and the erythrocyte surface. The results suggest that this is probably a transient event, the hemolysin becoming detached from the erythrocyte at some time after cell damage occurs. Because of the early release of potassium ions, it is likely that the hemolysin reacts with the erythrocyte membrane in the prelytic lag period. However, we could not demonstrate this by the fluorescent antibody method. The presence and intensity of fluorescence appear to be directly related to the quantity and duration of exposure to α -hemolysin. Failure to demonstrate specific fluorescence during the early stages of hemolysis may have been due to adsorption of a number of toxin molecules large enough to initiate cell damage, but too few to be seen.

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Trachoma and Inclusion Conjunctivitis Agents: Adaptation to HeLa Cell Cultures

Abstract. *The Mita-, Bour-, and Kami-strains of trachoma and inclusion conjunctivitis agents became adapted to serial passages in HeLa cell cultures after alternating passages between yolk sacs and tissue culture.*

The adaptation of trachoma and inclusion conjunctivitis (TRIC) agents to tissue culture is difficult (1). A Chinese strain of trachoma agent, TE-55, was adapted to HeLa cells by Stoker in 1959, as described by Collier (1). So far, this is the only strain of the trachoma agent adapted to growth in a cell line. Furness *et al.* (2) were able to adapt one British strain of inclusion conjunctivitis agent, LB-1, to HeLa cells, but failed in attempts to adapt the trachoma agent. Other investigators (3) described the cultivation of trachoma agents in tissue culture, but no mention was made of serial passage.

In our early attempts to adapt TRIC-agents to HeLa cells, the agents grew well in tissue culture during the first passage from yolk sacs, as shown by the formation of typical cytoplasmic inclusions. After several passages, however, the agents disappeared. After the TRIC-agents were alternated between yolk sacs and tissue culture, they became adapted to serial passage in tissue culture.

The Mita- and the Bour-strains of trachoma agent (4) became adapted to serial passage in tissue culture after 74 and 118 passages in yolk sacs, respectively. The Kami-strain of inclusion conjunctivitis agent (4) became adapted after 60 passages through yolk sacs. The tissue culture system consisted of HeLa cell monolayers grown in a yeast-lactalbumin hydrolysate (YLH) medium (5) containing 20 percent human serum in 250-ml bottles at 37°C.