NOTES

Specific and Nonspecific Staining in Detecting Staphylococcal Alpha Toxin on Erythrocytes by Immunofluorescence

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Specifically stained staphylococcal alpha toxin particles were detected on unstained and nonspecifically stained ghost membranes when rabbit erythrocytes were exposed to alpha toxin and stained with fluorescein isothiocyanate-labeled specific antitoxin. Fading of the nonspecific staining was observed and was attributed to the degradation of the membranes by alpha toxin.

A direct immunofluorescence method to detect staphylococcal alpha toxin on erythrocyte membranes and the observations made by this method are described in this report. Staphylococcus aureus strain 9A (3) and ATCC 10832 were used for alpha toxin production. The culture medium, in grams per liter, consisted of: Difco heart infusion, 25; Difco yeast extract, 3; L-arginine, 5; K2HPO4, 5.23. The medium was adjusted to pH 7.2, dispensed in 75-ml amounts into 250-ml Erlenmeyer flasks, sterilized at 15 psi for 15 min, inoculated with 0.1 ml of an 18-hr-old culture, gassed with CO2, and incubated at 37 C on a shaker for 72 to 96 hr. The cultures were centrifuged, the sediments were discarded, and the supernatant fluids were passed through HA-type Millipore filters. The filtrates, concentrated approximately fourfold by ultrafiltration through Diaflo type PM-10 membranes, were passed through Sephadex G-100 in 0.05 M NaPB with 0.5 M NaCl at pH 7.2 (2). Pooled, concentrated, active fractions were used in this project. The preparation of the phosphate-buffered saline solution (PBS) and 2% rabbit erythrocyte suspensions (RES), titration of the alpha toxin, determination of the hemolytic unit (HU), immunization of rabbits with alpha toxin, and titration of alpha antitoxin were previously described (3). The fluorescein isothiocyanate (FITC), purchased from Calbiochem, Los Angeles, Calif., was conjugated with globulin of alpha antitoxin by the method described by Cherry et al. (1).

Series of doubling dilutions of alpha toxin,
ranging from 2 to 256 HU per ml were prepared with ice-cold PBS. Tubes containing 2 ml of cold RES were centrifuged and placed in ice baths after supernatant fluids were discarded. Each erythrocyte sediment received 2 ml of one of the toxin dilutions. The cell-toxin mixtures were held in ice baths for 5 min. The cells were washed twice each time with 10 ml of cold PBS by centrifugation at 0 °C for 5 min at 15,000 × g. The cell sediments received three drops of FITC-labeled alpha antitoxin, were incubated at 37 °C for 10 min, cooled in ice baths, and washed again. Several drops of buffered glycerol (1) were added. Wet mounts were examined with a Zeiss fluorescence microscope for normal erythrocytes, ghosts, and fluorescence. The usual controls were included.

Hemolysis did not occur in any of the tubes until the temperature was raised. Two fluorescent substances were observed. Both were associated with erythrocyte ghosts. One substance, located in the ghost membranes, stained nonspecifically (5). The ghost cells appeared as fluorescent rings (Fig. 1) and were not detected until hemolyzed erythrocytes were present. The second substance was scattered over the membrane as fluorescent particles (Fig. 2). These were first found on ghosts in sediments of erythrocytes treated with 4 to 8 HU of alpha toxin and stained with FITC conjugates of specific antitoxin. The frequency of ghosts with fluorescent particles and the average number attached to each cell increased with toxin concentration. On the other hand, the initially brilliant fluorescent membranes faded rapidly and eventually disappeared. Clusters of fluorescent particles survived outlining the remains of disintegrating ghosts (Fig. 2).

The fluorescent particles were specifically stained clusters of alpha toxin adsorbed at 0 °C and remained on the membranes after the cells were washed and hemolyzed. The fading of the membrane fluorescence was a continuation of the degradation of the membrane by alpha toxin after the release of hemoglobin. Klainer et al. (4) also demonstrated alpha toxin on erythrocyte membranes by using an indirect method. Unfortunately, it could not be determined from their report whether the fluorescences they observed were similar to those described in this paper.

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LITERATURE CITED