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Electron Microscopic Localization of Alpha Toxin Within the Staphylococcal Cell by Ferritin-Labeled Antibody

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Highly purified staphylococcal alpha toxin has been used to produce monospecific anti-alpha antibody in rabbits. Gamma globulin prepared from the serum of these rabbits was coupled with ferritin by using toluene diisocyanate. Staphylococcal cells which had been disrupted by two passages through an LKB X-press were treated with this conjugate. Electron microscopic examination of this material showed alpha toxin or an antigenically mature precursor located on the cytoplasmic membrane. The possible function of alpha toxin in this situation is discussed.

In most studies of staphylococcal virulence, the lysins, first differentiated in 1935 (5), have been assumed to be weapons of offense. However, for more than 30 years (4) a minority of authors have taken the view that alpha toxin (and other toxins) may be a functioning component of the staphylococcal cell which unintentionally can affect a range of host cells. For a number of reasons discussed later, this minor view seemed plausible, and this led us to look for alpha toxin within the staphylococcus.

MATERIALS AND METHODS

Ferritin. Ferritin was prepared from fresh horse spleen by a modification of the method of Granick (6). The ferritin solution was finally lyophilized and stored dry at −10 C.

Examination in the electron microscope confirmed the characteristic shape of the ferritin particles (Fig. 2, 3, 4).

Anti-alpha gamma globulin. Several rabbits were injected with purified alpha toxin (3). The toxin was allowed to aggregate by standing at 4 C for several weeks, and the aggregate was suspended in distilled water. Equal amounts of this suspension and Freund's complete adjuvant were emulsified by ultrasonic agitation, the final concentration of toxin being approximately 1.5 mg per ml of emulsion. Injections were given at weekly intervals, each treatment consisting of five subcutaneous injections of 0.2 ml at five different sites. The course of antibody production was followed by gel diffusion. Gamma globulin was prepared from the pooled serum of several rabbits by the method of Nichol and Deutsch as described by Kabat and Mayer (8).

Coupling. Ferritin and gamma globulin were coupled by the method of Singer and Schick (12) with the modification that the coupling agent (toluene diisocyanate) was emulsified into the phosphate buffer by ultrasonic agitation immediately before chilling to 0 C and the addition of ferritin. Toluene diisocyanate is solid at 0 C and therefore impossible to couple effectively unless its surface area is greatly increased. A 5-ml amount of 1.5% (w/w) ferritin solution (containing 75 mg of ferritin) was coupled to a similar volume and concentration of gamma globulin.

Gel diffusion between purified alpha toxin and coupled antibody showed only one line of precipitation with the red-brown ferritin stain confined to the antibody side. This test also confirmed that the coupled antibody was still immunologically active.

Disruption and staining of cells. Wood 46 staphylococci centrifuged from 24-hr-old cultures used in alpha toxin production were stirred to a thick paste in 7.5% NaCl-phosphate buffer (*p*H 7.0). The paste was poured into the barrel of the 5-ml LKB X-press, frozen to −30 C, and passed twice through the aperture. The frozen plug was then thawed into phosphate-NaCl buffer, stirred, and then centrifuged.

A ferritin antibody solution which had been dialyzed overnight against 0.1 M $(NH_4)_2CO_3$ (*p*H 8.5) was run on to half the centrifuged pellet, and ferritin solution treated in a similar manner was added to the other half. After standing for 0.5 hr at room temperature, each preparation was centrifuged.

Fixation and embedding. Small portions of each pellet were fixed for 1 hr in 2.5% glutaraldehyde and postfixed in 1.6% OsO_4 with several changes of plain buffer between. Each reagent was dissolved in phosphate buffer (*p*H 7.4) containing 7.5% NaCl, and this solution was also used for washing. It has been shown that *Staphylococcus aureus* spheroplasts are isotonic with 24% NaCl (11). However, it was found that glutaraldehyde became milky if the salt concentration exceeded 7.5%. Preparations of cell fragments made with buffer alone did not show the same degree

of structural preservation as those made in buffer with 7.5% NaCl. For these reasons, buffer with 7.5% NaCl was used as the standard buffer for all procedures. Pieces of fixed material were then dehydrated in graded ethanol and embedded in Epon 812. Thin sections were cut in a Porter-Blum MT2 ultramicrotome and examined in a Hitachi HU 11E electron microscope. Sections were examined either unstained or double stained with saturated aqueous solution of uranyl acetate and alkaline lead citrate.

RESULTS

Preliminary studies showed that two passages through the LKB X-press gave a range of fragments from intact cells to small cytoplasmic pieces. Many cell walls with and without cytoplasmic membranes lining their inner surface were seen as were also wall-less cells with and without a limiting membrane surrounding them. Many of the cell wall ghosts contained within them a membrane which in parts was adherent to the inner surface of the wall but which elsewhere had rolled loosely upon itself into open spirals and vesicles (Fig. 4).

Ferritin-treated fragments. Samples incubated with ferritin alone showed no binding of ferritin particles to any of the cytoplasmic structures. In a few regions, some binding of amorphous electron-dense material varying greatly in size and shape was seen associated with membranes. These particles (Fig. 1) lacked the uniform size and typical appearance of ferritin (Fig. 2–4). As they could be found neither in samples of ferritin nor in the sections treated with ferritin-antibody conjugate, it must be assumed that disrupted staphylococcal cell fragments have the ability to dissociate some iron from apoferritin and to bind some of the liberated iron to cytoplasmic membranes. Binding of iron to the cytoplasmic membranes of mammalian liver (1) and intestinal cells (C. L. Kimber, T. M. Mukherjee, and D. J. Deller, Blood, *in press*) is known.

Ferritin-antibody conjugate-treated fragments. Particles, proven by their uniform size (5.5 nm) and shape to be ferritin, were seen in marked concentration attached to membranes and to no other structure (Fig. 2–4). It was in sections unstained with either uranyl acetate or lead citrate (Fig. 2) that the magnitude of ferritin binding to the membranes was seen most clearly (cf. Fig. 1). Fragments of cell wall (Fig. 6) or cytoplasm (Fig. 7) showed no binding. Ghosts of dividing cells containing vesicular arrangements of cytoplasmic membranes however showed relatively less binding (Fig. 4) when compared with ghosts of nondividing cells (Fig. 2, 3). In some instances, membranes of dividing cells showed no binding of ferritin particles whatsoever (Fig. 5). It seems likely from these observations that alpha toxin is not produced uniformly throughout the life cycle of an individual staphylococcal cell.

DISCUSSION

The ability of staphylococcal culture filtrates to lyse a wide variety of cells, particularly red cells of many species, has, since the earliest reports of these effects, usually been discussed in reference to the cell membrane. As reports on purification procedures for alpha toxin have never described a nonlytic, specific-antibody-combining material, it seems most likely that the agent on the membrane combining with ferritin antibody is definitely alpha toxin.

Alpha toxin was revealed in high concentration only on those cytoplasmic membranes in which the inner surface was exposed to ferritin antibody, and no ferritin was seen associated with the cell wall. Alpha toxin is, therefore, formed just under the limiting membrane or, if formed deeper within the cell, travels relatively quickly to the inner surface of the membrane, there to be loosely bound or temporarily blocked from passing across the membrane. Once across the membrane, the toxin must again travel quickly through the cell wall and on into the surrounding medium.

Bernheimer and Schwartz (2) showed that a strain giving high yields of alpha toxin produced 2% of its own dry weight as this single protein. As the cell membrane comprises only 6% of the volume of the whole cell and alpha toxin is localized on this organelle, it is apparent that this protein forms a considerable part of the cell membrane. This suggests that alpha toxin has a functional role on the staphylococcal membrane, as a large amount of nonfunctional protein in association with the membrane would be an embarrassment to the cell.

Experiments (*in preparation*) have been performed in our laboratory in which washed staphylococcal cells were grown in culture with and without the addition of purified alpha toxin. Although the difference in growth rate between such cultures was slight, the cells to which alpha toxin was added always grew faster than the control.

Also more consistent with a metabolic role for toxins rather than a virulence role are the following. (i) Work of Kapral and Li (9) in which mutant substrains of virulent staphylococci were shown to possess or lose virulence unrelated to coagulase production, the traditional sine qua non of pathogenicity. (ii) The staphylococcus is a very common commensal which confers some benefit on man (11), yet most of these organisms produce alpha lysin. (iii) Pure alpha lysin is not a good human antigen (*personal observation*). (iv)

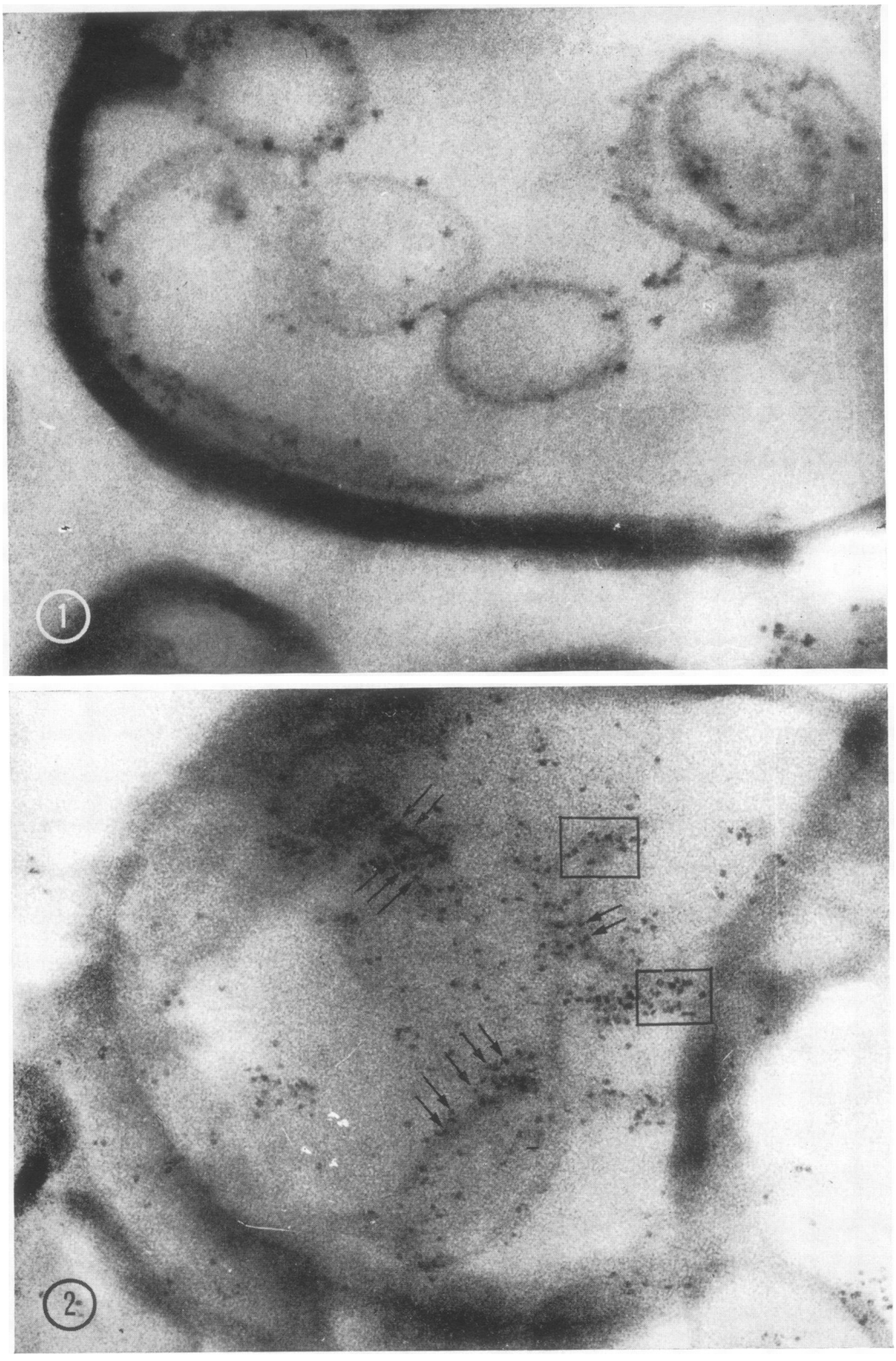
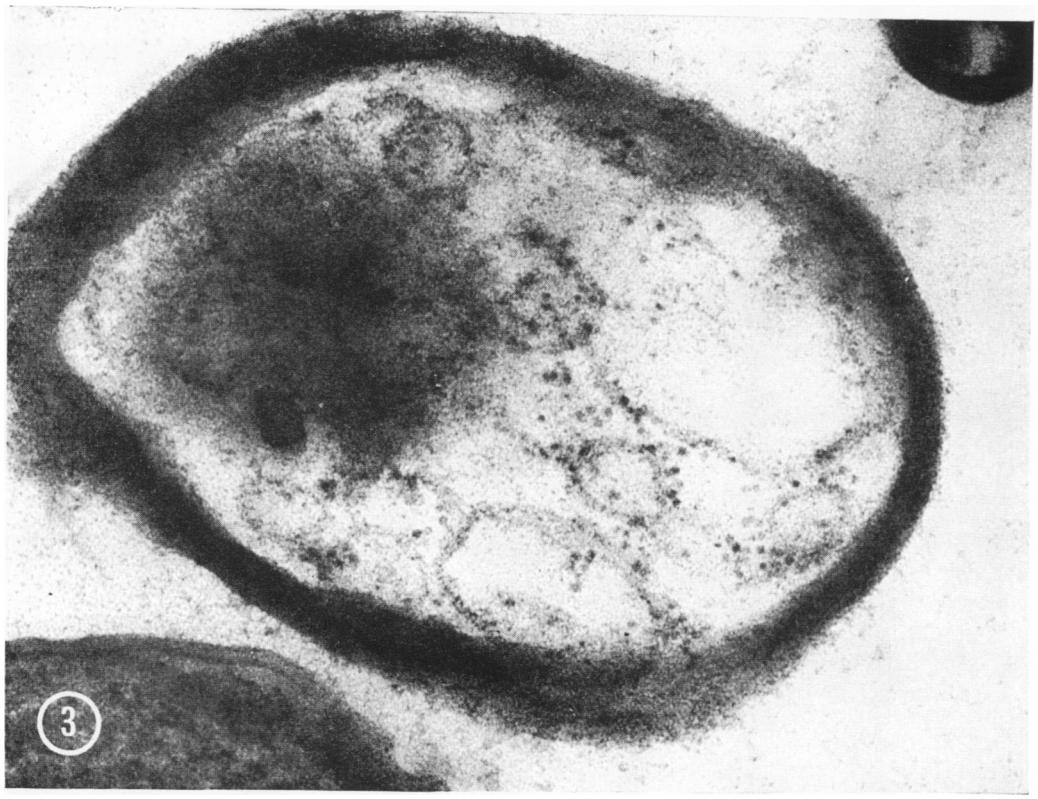


FIG. 1. *Electron micrograph of an unstained section of a pellet incubated with ferritin alone showing a portion of cell wall with several membrane vesicles. Note the presence of electron-dense deposits in association with the membranes. These deposits vary considerably in shape and size and probably represent iron extracted from apoferritin since none of these have the distinctive appearance of ferritin (cf. Fig. 2).* × *170,000.*

FIG. 2. *Unstained section of pellet incubated with ferritin antibody conjugate showing the enormous binding of ferritin particles to the membrane vesicles (arrows). That these are ferritin particles is proved by their uniform size distribution (*∼ *5.5 nm) and typical appearance of four subunits as seen in regions marked by boxes.* × *190,000.*



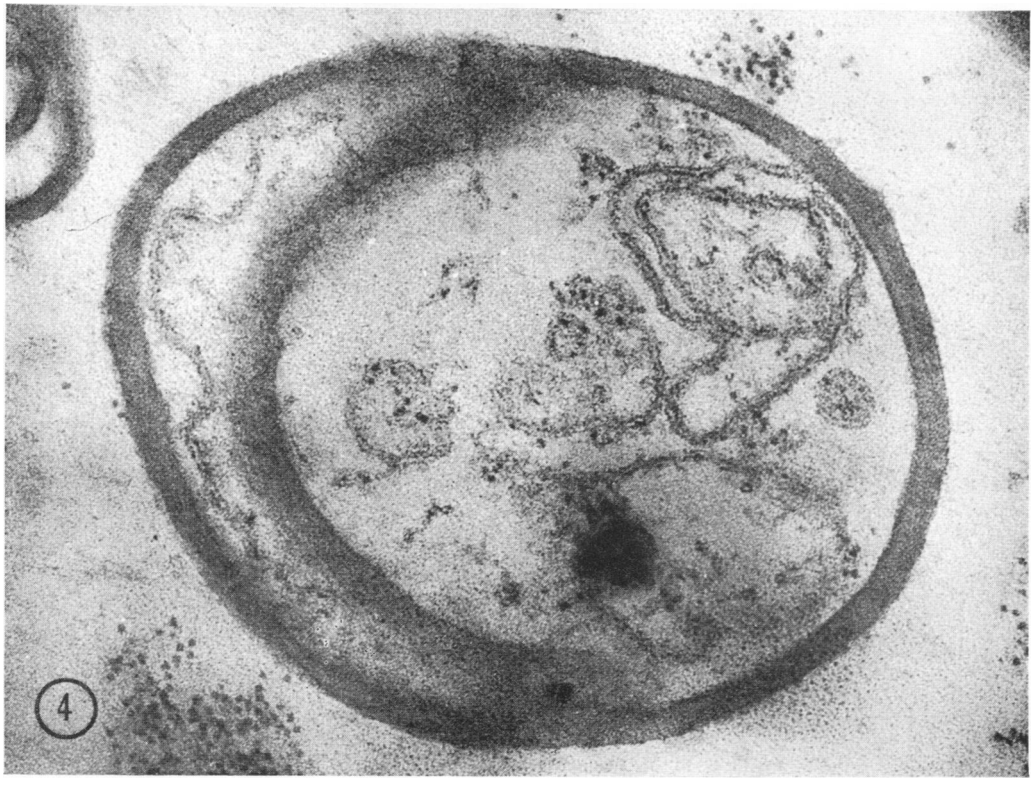
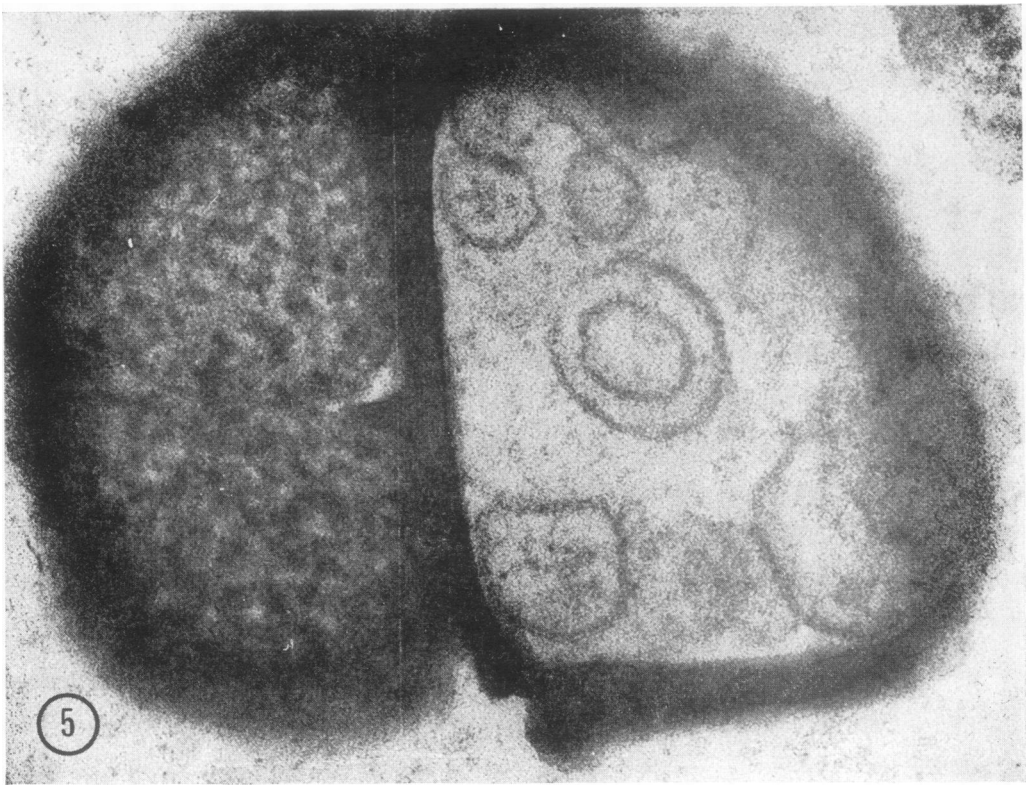
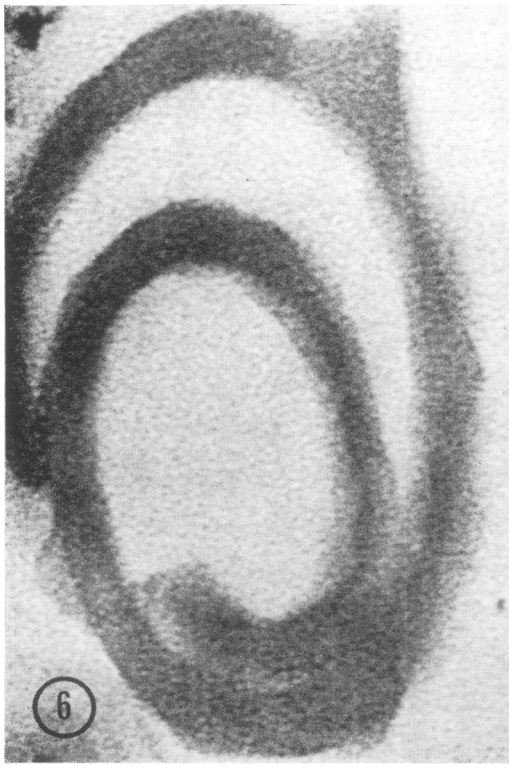


FIG. 3. *Section from a pellet incubated with ferritin-antibody-conjugate and stained with uranyl acetate and lead citrate showing the magnitude of ferritin binding only to the membranes. No particles are seen in relation to the cell wall.* × *162,000.*

FIG. 4. *Dividing cell ghost from the pellet as in Fig. 3 showing attachment of ferritin particles to membranes. The concentration of attachment is, however, small in comparison to Fig. 3. Some collections of ferritin particles can be seen in the surrounding medium, but this was not frequently seen and could not be associated with any remnants of cytoplasmic structures. No ferritin binding is observed on the cell wall.* × *140,000.*





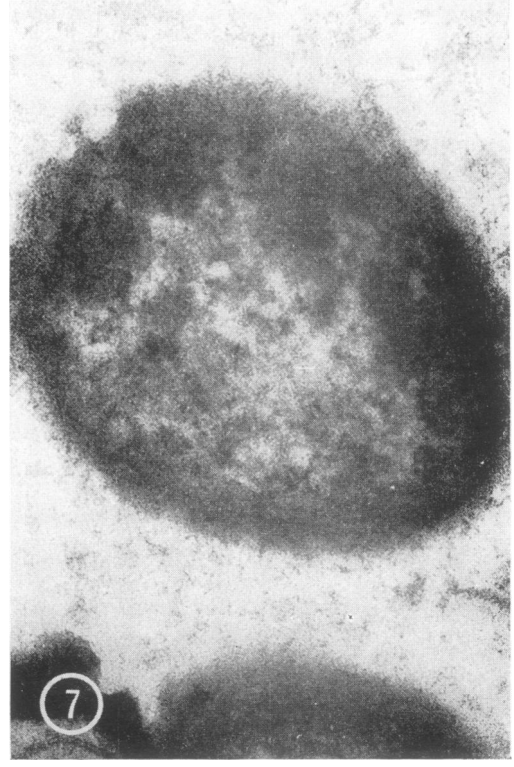


FIG. 5. *Dividing cell ghost with partial absence of cytoplasmic material showing no ferritin attached to the membranes. Specimen treated as in Fig. 3 and 4.* × *145,000.*

FIG. 6. *Remnants of cell wall showing no binding of ferritin. Specimen as in Fig. 2.* × *155,000.*

FIG. 7. *Nearly a spheroplast, but without a considerable portion of the cell membrane showing absence of ferritin binding anywhere in the cytoplasm. (Sample as in Fig. 3 and 4).* × *120,000.*

Anti-alpha lysin antibody levels correlate poorly with staphylococcal disease.

The known effects of alpha toxin on the permeability of a variety of cell membranes taken with our present observations tempt us to suggest that the role which alpha toxin may have within the staphylococcus is the modification or control (or both) of membrane permeability.

Consistent with this thesis is the observation of Hallander et al. (7) who showed that the addition of subinhibitory amounts of penicillin to growing staphylococcal cultures resulted in an increased yield of alpha toxin but decreased the yields of other exotoxins. If the principal function of alpha toxin is to modify the permeability of the cell membrane, then it is reasonable to postulate that a mechanism controlling production of alpha toxin must exist and that this mechanism would be sensitive to the concentration of alpha toxin on the membrane at any given time. That some control mechanism exists is supported by our observation that the amount of alpha toxin on the cytoplasmic membrane seems to vary with the age of the cell. The concentration of alpha toxin on the membrane reflects the difference between the rate of formation and the rate of loss, across the cell wall, to the surrounding medium. A defective cell wall resulting from subinhibitory concentrations of penicillin may increase the rate of leakage of alpha toxin from the membrane and thus decrease the concentration of alpha toxin on the membrane. Still assuming a permeability function for alpha toxin, this would have two effects. The permeability of the membrane to other substances (i.e., other exotoxins and nutrients) could be decreased and the control mechanism would be set to produce large amounts of alpha toxin.

It was this interpretation of Hallander's findings which led us to look for alpha toxin on the staphylococcal cytoplasmic membrane; finding it on this site reinforces the validity of the concept that alpha toxin modifies or controls (or both) the permeability of the staphylococcal cell membrane.

Some authors have posed the question "If alpha toxin is such a powerful lytic substance disrupting the membranes of a wide variety of cells, how is it that the cell which synthesizes the toxin is not itself affected." There seems to us a very satisfying and simple elegance in the notion that alpha toxin does have an effect on the staphylococcal membrane and that this may be precisely "why it is made."

ACKNOWLEDGMENTS

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