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PHOTOLABELING OF STAPHYLOCOCCAL α-TOXIN FROM WITHIN RABBIT ERYTHROCYTE MEMBRANES

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SUMMARY: Intrinsic membrane proteins of rabbit red blood cells were labeled with the photoreactive amphipatic reagent 12-(4-azido-2-nitrophenoxy)stearoyl (1-^{14}C) glucosamine, which inserts into the hydrophobic membrane region and generates a reactive nitrene upon ultraviolet irradiation. Photolabeling of membrane-bound staphylococcal α-toxin after lysis of probe-treated rabbit red blood cells by this toxin implies its penetration into the hydrophobic region of the outer leaflet of the membrane. In contrast clostridial θ-toxin and staphylococcal δ-toxin were not labeled, but extraction of intrinsic membrane proteins by δ-toxin was evidenced.

Staphylococcus aureus produces four hemolysins of which α-toxin is considered to be a major virulence factor (see ref. 1 for review). This surface active protein consists of a single 34.000-dalton polypeptide (2), and has lethal, dermonecrotic and membrane-damaging properties (1).

Rabbit red blood cells (RRBC) are 100-1000 times more sensitive to α-toxin than erythrocytes from any other species, probably due to the presence in RRBC of a membrane receptor, which binds α-toxin with high affinity (3,4). RRBC-lysis with α-toxin proceeds via three stages: (i) binding of toxin to the cell membrane, (ii) K^+-ion-leakage, and (iii) hemoglobin leakage (3). The intramembrane events leading to lysis are not well understood at the molecular level, but there is a large body of evidence consistent with a mechanism involving general membrane perturbation by hydrophobic interaction of the toxin with the lipid

ABBREVIATIONS: Rabbit red blood cells: RRBC, 12-(4-azido-2-nitrophenoxy) stearoyl (1-^{14}C) glucosamine: 12-APS-GlcN, phosphate buffered saline: PBS.

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bilayer (2,5,6,7). However, it is not clear whether this interaction involves actual membrane penetration of the toxin.

To investigate the possible penetration of α-toxin in RRBC membranes we used a photoreactive probe, the amphipatic glycolipid 12-(4-azido-2-nitrophenoxy) stearoyl-(1-^{14}C)glucosamine (12-APS-GlcN). This molecule spontaneously inserts in membranes, and upon UV-irradiation generates a reactive nitrene group which labels membrane proteins at approx. 13Å from the cell surface, i.e., in the hydrophobic part of the outer lipid bilayer (8,9). The photolabeling pattern of RRBC membrane proteins obtained after hypotonic lysis was compared to the patterns resulting after toxin-induced lysis. We present herein the first evidence that α-toxin does insert into an intact cell membrane.

MATERIALS AND METHODS: Chemicals. (1-^{14}C) glucosamine was purchased from NEN Chemicals GmbH, Dreieich, FRC.

Photoreactive probe. 12-(4-azido-2-nitrophenoxy)stearoyl-(1-^{14}C)-glucosamine (12-APS-GlcN) was synthesized according to Wisnieski et al. (9) with some modifications, as will be described separately (Jolivet-Reynaud et al., in preparation). The specific radioactivity of the probe was 50 Ci/mole.

Toxins. Alpha-toxin was purified from S.aureus strain Wood 46 according to Wadström (10). S.aureus δ-toxin was purified according to Kreger et al. (11) and C.perfringens θ-toxin (perfringolysin O) according to Smyth (12). The specific activities against RRBC of these toxins were 25.000 (α), 100 (δ) and 55.000 (θ) hemolytic units per mg of protein.

Incorporation of probe, toxin treatment and photolabeling. RRBC suspensions were prepared in phophate buffered saline, pH 6.8 (PBS) as previously described for sheep RBC (13). Four ml of intact RRBC at a final concentration of 10% were treated for 60 min at 37°C with 0.4-1.0x10^{-2} μCi of probe per μg of membrane protein. The ethanolic probe solution never exceeded 1% of the volume of the final suspension. Probe-treated cells were centrifuged (200 x g, +4°C), the cell pellet was washed 4-5 times in 20-30 ml volumes of buffer and resuspended in PBS to a final concentration of 2.5%.

Equal amounts of the same pool of probe-treated cells (2 ml of RRBC suspension corresponding to 200 μg of membrane protein) were lysed by incubation for 15 min at 22°C with 200 μg of each toxin. Hypotonic lysis was induced under the same conditions using 5 mM sodium phosphate buffer, pH 8 (14). The resulting ghosts were centrifuged (20.000 x g, +4°C), washed once in 500 μl of hypotonic buffer, resuspended in 200 μl of buffer, and irradiated for 10 min with a lamp emitting at 360 nm (Mineralight Ultraviolet lamp, Ultra-Violet Products Inc., San Gabriel, Calif.). The irradiated ghosts were washed once, and solubilized in SDS-2-mercaptoethanol-buffer before application on gels. All operations involving the photoreactive probe were performed under red safety lights until after the UV-irradiation.

Sodium dodecylsulfate polyacrylamide gel electrophoresis. This was performed according to Laemmli (15) on continuous 7.5-20% gradient gels with a 3% acrylamide stacking gel and a 1% SDS/Tris/glycine running buffer. After electrophoresis the gels were stained with Coomassie blue R-250 and

prepared for fluorography according to Bonner and Laskey (16). Twice pre-flashed Kodak-XR-1 films were exposed to the gels for 7-15 days at -70°C before development.

RESULTS: Both staphylococcal α-toxin and clostridial θ-toxin bound to the RRBC membrane during hemolysis as seen from the protein bands incorporated at positions corresponding to the free toxin bands (Fig. 1, lanes 4,5 and 7,8). With the Coomassie blue staining staphylococcal δ-toxin was not visualized either alone (lane 10) or membrane-bound (lane 11) although the amount of toxin added caused complete lysis. The three toxins tested did not appear to alter the pattern of Coomassie blue stained membrane protein bands to any significant extent.

The corresponding fluorogram indicates that:
(i) In the hypotonically lysed ghosts there were two heavily labeled and two weakly labeled components, here referred to as A, B, C and D (Fig. 1, lane 1). (ii) Membrane-associated α-toxin (lane 3) migrated to roughly the

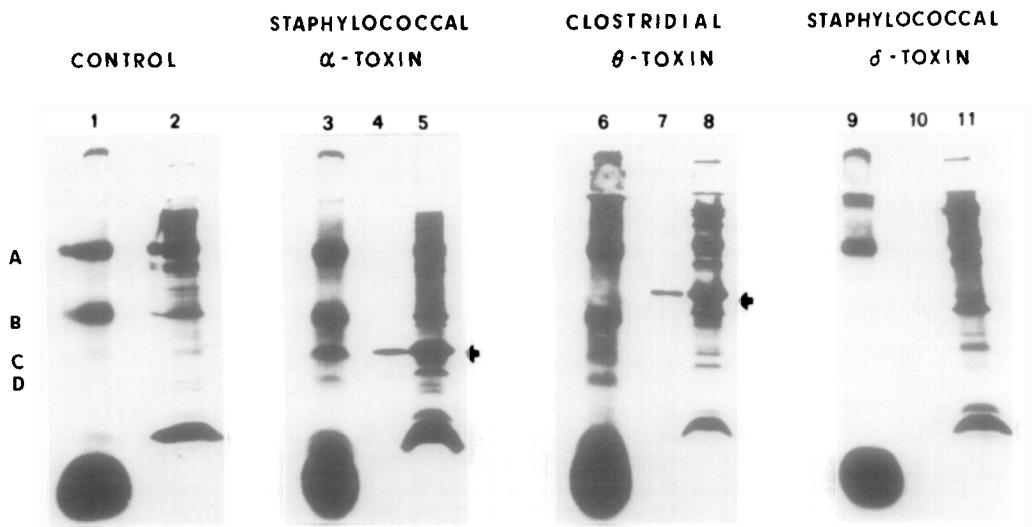

Fig. 1: Sodium dodecylsulfate polyacrylamide gel electrophoresis of RRBC ghosts after hypotonic lysis (control), lysis with α-toxin, θ-toxin and δ-toxin. RRBC were treated with probe, lysed by hypotonic buffer or by the toxins and further processed as described under Materials and Methods. Lanes 2, 5, 8 and 11: ghosts stained by Coomassie blue. Lanes 1, 3, 6 and 9 show the corresponding fluorograms. Lanes 4, 7 and 10: Coomassie blue stained free α-, θ- and δ-toxins respectively. Arrows indicate toxin location.

same position as band C. However, a large part of the label clearly corresponds to the toxin band, as seen from its shape (cf lane 5), and from its greater intensity as compared to band C in the control ghosts (lane 1). The intensity of bands A and B in α-toxin treated ghosts is similar to that of bands A and B in the control ghosts. (iii) No labeled component was found on the fluorogram (lane 6) in the position of the membrane associated θ-toxin (cf. lane 8). (iv) A possibly existing labeled δ-toxin band (in lane 9) might be masked by the probe-label at the bottom of the lane (lipidreacted + unreacted probe). This possibility was investigated by electrophoresis of δ-toxin-treated ghosts on a 25% polyacrylamide gel to allow for separation between δ-toxin monomer (Mr=2960) and probe (not shown). However, neither in this case could any δ-toxin be detected as associated to the ghosts. (v) The intensity of band A in the δ-toxin-treated ghosts is unchanged (lane 9), whereas the other three labeled bands have almost disappeared, as compared to the control ghosts, as well as to those treated with the other two toxins. The protein corresponding to the heavily labeled band B (lane 1) appears to havebeen virtually extracted during lysis with δ-toxin. This result was confirmed with another δ-toxin preparation (kindly supplied by Dr. John Freer) (data not shown).

DISCUSSION: Various kinds of photoreactive reagents which spontaneously insert into the hydrophobic region of natural and artificial membranes have recently been described (17,18,19). Such probes have been utilized to label intrinsic RBC membrane proteins (17), for the study of membrane attack by complement (20), and for analysis of the transmembrane dynamics of cholera toxin (8,9). The present work demonstrates that the amphipatic nitrene generating reagent 12-APS-GlcN can be successfully applied also for the study of penetration of membrane damaging toxins into the hydrophobic membrane region.

The main radioactive bands (A-C) in the hypotonically lysed RRBC correspond to regions containing known intrinsic proteins in human RBC. Band A corresponds to band 3 and PAS-1, band B to the 4.5 region and PAS-2, and

band C to PAS-3 (21). The weakly labeled band D does not correspond to any well-characterized protein. The amount of label is low in the regions containing known extrinsic proteins, i.e. band 1,2,5 and 6. In essence our findings are consistent with those of Bayley and Knowles (17) who labeled intrinsic membrane proteins in human RBC-ghosts with a lipophilic carbene-generating probe. The fact that in the control ghosts only intrinsic membrane proteins were labeled constitutes a valuable control for the intramembrane specificity of our probe.

The labeling of staphylococcal α-toxin, in comparison to non-labeling of the θ- and δ-toxins, strongly suggests that α-toxin penetrated into the hydrophobic region of at least the outer lipid layer of the RRBC-membrane. This finding is consistent with previous reports that α-toxin penetrates lipid monolayers (5), and that it causes changes in the hydrophobic fracture plane of erythrocytes and platelets (6,7). Our result also supports the more recent postulate that hexamers of α-toxin may form transmembrane pores in RBC treated with large amounts (150-200 μg) of the toxin (2).

Theta-toxin served as a valuable negative control, i.e., although being fixed to the membrane and causing complete lysis, it did not become labeled by the probe. Neither did it remove any intrinsic or extrinsic membrane proteins. This finding is consistent with the current view that θ-toxin induces lysis by interaction with membrane cholesterol, causing its sequestration and thereby membrane destabilization by impairment of normal cholesterol - phospholipid interactions (22).

Staphylococcal δ-toxin was included to serve as a positive control, since it is more surface-active than α-toxin and, according to Colacicco and co-workers "readily inserts itself into hydrophobic membrane structures" (23). Moreover Freer and Birkbeck (24) recently postulated that δ-toxin monomers might associate into hexameric complexes in the membrane, with formation of transmembrane pores. Our results with δ-toxin are difficult to interpret since the toxin could not be visualized in the gels. However, as our results reflect the final outcome of the lytic process the possible for-

mation of pores in an early stage of toxin-membrane interaction can not be ruled out. The finding that δ-toxin appears to have caused the removal of some membrane protein is consistent with the view that it behaves as a "detergent-like" molecule (25,26).

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REFERENCES

1. Rogolsky, M. (1979) Microbiological Reviews 43, 320-360.
2. Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T., and Wellensiek, H.J. (1981) J. Cell. Biol. 91, 83-94.
3. Harshman, S. (1979) Mol. Cell. Biochem. 23, 143-152.
4. Maharaj, I., and Fackrell, B. (1980) Can. J. Microbiol. 26, 524-531.
5. Buckelew, A.R., and Colacicco, G. (1971) Biochim. Biophys. Acta 233, 7-16.
6. Bernheimer, A.W., Kim, K.S., Remsen, C.C., Antanavage, J., and Watson, S.W. (1972) Infect. Immunity 6, 636-642.
7. Freer, J.H.,Arbutnott, J.P., and Billcliffe, B. (1973) J. Gen. Microbiol. 75, 321-332.
8. Wisnieski, B.J., and Bramhall, J.S. (1979) Biochim. Biophys. Res. Commun. 87, 308-313.
9. Wisnieski, B.J., Shiflett, M.A., Mekalanos, J., and Bramhall, J.S. (1979) J. Supramolec. Structure, 10, 191-197.
10. Wadström, T. (1968) Biochim. Biophys. Acta 168, 228-242.
11. Kreger, A.S., Kim, K.S., Zaboretzky, F., and Bernheimer, A.W. (1971) Infect. Immunity, 3, 449-465.
12. Smyth, C.J. (1975) J. Gen. Microbiol. 87, 219-238.
13. Alouf, J.E., and Jolivet-Reynaud, C. (1981) Infect. Immunity, 31, 536-546.
14. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
15. Laemmli, U.K. (1979) Nature (London), 227, 680-685.
16. Bonner, W.N., and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88.
17. Bayley, H., and Knowles, J.R. (1980) Biochemistry, 19, 3883-3892.
18. Bisson, R., and Montecucco, C. (1981) Biochem. J. 193, 757-763.
19. Chakrabarti, P., and Khorana, H.G. (1975) Biochemistry, 14, 5021-5033.
20. Hu, V.W., Esser, A.F., Podack, E.R., and Wisnieski, B.J. (1981) J. Immunol, 127, 380-386.
21. Steck, T.L. (1974) J. Cell. Biol. 62, 1-19.
22. Smyth, C.J., and Duncan, J.L. (1978) Bacterial Toxins and Cell Membranes (J. Jeljaszewicz and T. Wadström, eds.). pp. 129-183. Academic Press, New York.
23. Colacicco, G., Basu, M.K., Buckelew, A.R., and Bernheimer, A.W. (1977) Biochim. Biophys. Acta, 465, 378-390.
24. Freer, J.H., and Birkbeck, T.H. (1982) J. Theor. Biol. 94, 535-540.
25. Bernheimer, A.W. (1974) Biochim. Biophys. Acta, 344, 27-50.
26. Thelestam, M., and Möllby, R. (1979) Biochim. Biophys. Acta, 557, 156-169.