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Oligomerisation of cell-bound staphylococcal α-toxin in relation to membrane permeabilisation

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We have studied the kinetics of staphylococcal α-toxin oligomerisation in relation to membrane permeabilisation, using as targets cultured adrenocortical Y1 cells, rabbit red blood cells (RRBC), human platelets, and liposomes prepared of lipids extracted from platelets. After isolation of membranes from toxin-treated cells, oligomeric toxin was detected (i) by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography or Western blotting, and (ii) by electron microscopy of negatively stained specimens. α-Toxin was found to oligomerise on all membranes independently of the temperature. On RRBC and Y1 cells most of the membrane associated toxin appeared converted to the oligomeric form. Hexamers were always present along with membrane permeabilisation. However, hexamers were also detected at conditions when membrane permeabilisation did not occur; at low temperature, in the presence of high concentrations of Ca^{2+}, and after pretreatment of cells with concanavalin A (Con A). Addition of a neutralising monoclonal antibody (MAb) to cell-bound toxin collected it into aggregates much larger than the hexamers. By contrast hexameric toxin remained after addition of a non-neutralising MAb. Our data suggest that the active toxin species is not monomeric, and support the hypothesis that α-toxin permeabilises membranes by forming hexameric protein-lined transmembrane channels.

Introduction

α-Toxin, a 33 kDa protein, is secreted by most strains of *Staphylococcus aureus* and is regarded as a major virulence factor of this bacterium. The toxin is lethal, dermonecrotic, cytotoxic and hemolytic. Central to all these biological effects is most probably the membrane damaging capacity of α-toxin (for reviews, see Refs. 1 and 2).

α-Toxin molecules undergo a transition from hydrophilic to amphiphilic form upon association with membranes [3,4], whereafter they are believed to penetrate as monomers into the membranes [4–6]. Following membrane association the toxin is aggregated to oligomers which can be isolated from membranes permeabilised by the toxin at 37°C and which are thought to be responsible for the membrane permeabilisation [1,6].

Recent studies on the mode of α-toxin action have focused largely on its interaction with artificial membranes [4,7–9] which are useful because the disturbing influence of naturally occurring membrane proteins and carbohydrates is removed. However, such model systems represent simplifications of the plasma membranes of living cells, which are the natural targets of α-toxin and therefore should be studied in parallel. Thus, among blood cells particularly rabbit erythrocytes (RRBC) [6], human platelets [10] and monocytes [11] have been studied. Among cultured cells mouse adrenocortical Y1 cells are highly sensitive and have therefore been used as a suitable model [12] while many other cell types respond to higher concentrations of the toxin (see Table 1 in Ref. 2).

Our previous findings concerning α-toxin interaction with Y1 cells [12] were consistent with the view that monomeric toxin associates with the cell membrane at

Abbreviations: RRBC, rabbit red blood cell; TBS, Tris-buffered saline; HBSS, Hank's balanced salt solution; Con A, concanavalin A; HU, hemolytic units; PAGE, polyacrylamide gel electrophoresis; MAb, monoclonal antibody; cpm, counts per min; Na-PTA, sodium phosphotungstic acid.

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low temperature and the toxin becomes membrane damaging after undergoing a conformational change at 37°C. We assumed this change to involve formation of toxin oligomers on or in the membrane, and more recently showed by SDS-PAGE that hexamers could indeed be isolated from the plasma membranes of Y1 cells treated with ^{3}H-labelled toxin [13]. Unexpectedly it was also found that α-toxin fragments formed oligomers of similar size upon binding to Y1 cells but without increasing the plasma membrane permeability. This observation prompted the present work which aimed at obtaining a clearer understanding of the role of oligomers for the membrane permeabilisation induced by α-toxin in sensitive cultured mammalian cells.

Materials and Methods

Chemicals. Trypsin, sera and cell culture media were obtained from Flow Laboratories, Irvine, Scotland. Aquasol Universal LSC Cocktail and [5-^{3}H]uridine (specific activity 26.7 Ci/mmol) were from NEN Research Products, Boston, MA, U.S.A., Bolton-Hunter reagent (*N*-succinimidyl[2,3-^{3}H]propionate) was purchased from Amersham International, Amersham, U.K. Concanavalin A (Con A) EC 2010 and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO. Other chemicals were from E. Merck AG, Darmstadt, F.R.G. and KEBO, Stockholm, Sweden. All chemicals were of analytical grade. Growth medium = Eagle's medium with 10% newborn bovine serum. Elution buffer = 0.05 M sodium phosphate buffer (pH 7.5) with 0.25% gelatin. TBS = Tris-buffered saline, i.e., 0.9% NaCl with 0.02 M Tris (pH 7.4).

Polyclonal, monoclonal and detection antibodies. Antibodies against purified α-toxin were raised in rabbits and purified according to standard methods [14]. The monoclonal antibodies 12E and 10D have been described [15]. An alkaline phosphatase-coupled anti-mouse immunoglobulin conjugate from Dakopatts A/S, Glostrup, Denmark was used for MAb-detection.

Preparation and radiolabelling of toxin and fragments. α-Toxin was prepared from *S. aureus* strain Wood 46 and purified by isoelectric focusing and gel filtration as previously described [16,17]. Purified toxin preparations contained 30000–50000 hemolytic units (HU) per mg protein. Tryptic toxin fragments were generated by digestion of native α-toxin (1 mg in 1.5 ml TBS) with 30 μg trypsin for 4 h at 37°C (pH 8.0). The reaction was terminated by the addition of 30 μg lima bean trypsin inhibitor. These conditions were found to be optimal for production of two stable fragments according to SDS-PAGE (shown in Refs. 13 and 18). Toxin and fragments were labelled with Bolton-Hunter reagent (*N*-succinimidyl[2,3-^{3}H]propionate) as described [19]. The specific radioactivities of the labelled preparations ranged from approx. 1.0 to 1.2 μCi/μg protein.

Blood cells and assay of hemolysis. Human platelets were obtained by mixing fresh blood with 3.8% sodium citrate (4 : 1) followed by centrifugation (15 min, 150 × *g*, 22°C). The supernatant containing platelet-rich plasma was further centrifuged (30 min, 1800 × *g*, 22°C). The sedimented platelets were suspended in TBS (pH 7.0) and the centrifugation was repeated. The pelleted platelets were resuspended in TBS and incubated 1 : 2 with α-toxin. The hemolytic activity of the toxin was assayed on microtiter plates with rabbit red blood cells (RRBC) as previously described [20]. The titer was expressed as the inverted value of the highest dilution causing 50% hemolysis.

Cultivation of cells, membrane isolation and assay of membrane damage. Mouse adrenocortical (Y1) tumour cells were cultivated in Ham's F10 medium as previously reported [17]. Cells cultivated to confluency in 6-well polystyrene plates (A/S Nunc, Roskilde, Denmark) were treated with α-toxin in 0.5 ml/well of elution buffer, TBS or growth medium whereafter the cells were rinsed twice with Hank's balanced salt solution (HBSS). Each well contained approximately $0.5 \cdot 10^6$ cells and the cells from six wells were pooled to give a total of approx. $3 \cdot 10^6$ cells per experiment. The membranes were isolated as previously described [13] except that the whole procedure was performed at 0–4°C. The final membrane pellet was dissolved in 120 μl of sodium dodecylsulphate (SDS) preparation buffer [21] and the radioactivity in 10 μl was determined by liquid scintillation. Half (50 μl) of the remaining sample was boiled for 5 min, before electrophoresis in SDS according to Laemmli [21] on continuous 7–20% polyacrylamide gradient gels with a 3% acrylamide stacking gel. Gels were stained with Coomassie blue R-250. For autoradiography 1 M sodium salicylate + 0.5% glycerol was used as enhancer during the film exposure, which was carried out at −70°C for the time periods indicated. For Western blotting the proteins were transferred from gels to nitrocellulose membranes and the proteins reacted with monoclonal anti-α-toxin antibody.

Damage to the plasma membranes of growing cells was measured as release of a radioactive cytoplasmic nucleotide marker from cells prelabelled with [5-^{3}H]uridine and treated with toxin. The nucleotide release was expressed as the percentage of a maximal release [17]. All experiments were conducted at least twice with duplicate samples.

Electron microscopy. Human platelets were prepared as described above. Lipids were extracted by dissolving the platelet membranes in chloroform/methanol (1 : 1.2, v/v). The lower phase obtained after centrifugation at 3000 × *g* for 1–2 min was withdrawn and brought to dryness under a stream of nitrogen. The residual total lipid was dissolved in TBS (1 mg/ml).

After incubation of either α-toxin or tryptic fragments of the toxin with the desired target negative

staining was performed instantly either with a 1% solution of Na-phosphotungstic acid (Na-PTA) at pH 7 or with a mixture of glucose and Na-PTA at final concentrations of 0.5%, respectively.

Electron microscopy was performed under controlled dose conditions with a Philips EM301. Negatively stained orthorombic catalase crystals were used to calibrate the magnification of the electron microscope.

Results

Oligomerisation of α-toxin on membranes at low temperature

Confluent monolayers of Y1 cells were exposed to ^{3}H-toxin for 15 min at 0°C whereafter the cells were washed and incubated in medium at 37°C. Plasma membranes were isolated from the cells directly after the binding step as well as after different time periods at 37°C. As we had noted previously [13], the toxin could be detected in the membrane fraction in hexameric (as well as monomeric) form when the binding step was followed by incubation at 37°C (Fig. 1). Surprisingly, hexamers had been formed already during the binding step at low temperature and the degree of oligomerisation did not increase upon incubation at 37°C. Parallel experiments with nucleotide labelled Y1 cells showed that up to 32 μg/ml toxin did not permeabilise the membranes during incubation at 0°C for up to 30 min (data not shown, but no single value was higher than 2% of the maximal release).

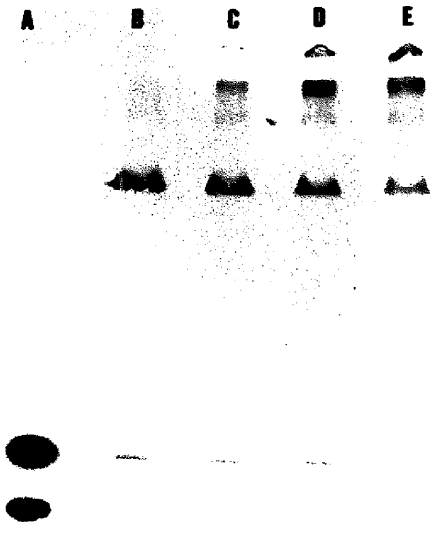

Fig. 1. Autoradiogram showing oligomerisation of α-toxin on Y1 cell membranes. Y1 cells were exposed to 16 μg/ml ^{3}H-toxin in elution buffer for 15 min at 0°C whereafter the toxin was removed, and the cells washed and further incubated at 37°C in growth medium. After different time periods the plasma membranes were isolated and processed as detailed in Materials and Methods. The membrane pellets were not boiled before SDS-PAGE of the membrane proteins. Lane A: ^{3}H-toxin without Y1 cells; lanes B to E: ^{3}H-α-toxin/Y1 cell membranes after post-incubation at 37°C for 0 min (B), 5 min (C), 15 min (D) and 30 min (E). The absolute cpm values were 971, 1095, 1005 and 637 in 10 μl samples of the solutions in lanes B, C, D and E, respectively. The volumes laid on the gel were adjusted to give approx. 2900 cpm in each lane. The film exposure time for autoradiography was 10 weeks. The low-molecular weight degradation product seen in lane A (and in some of the other figures) is present in some of our α-toxin preparations but apparently does not influence the oligomerisation pattern. The high-molecular weight aggregate on top of lanes D and E should not be confused with the hexamers seen lower down in lanes B to E.

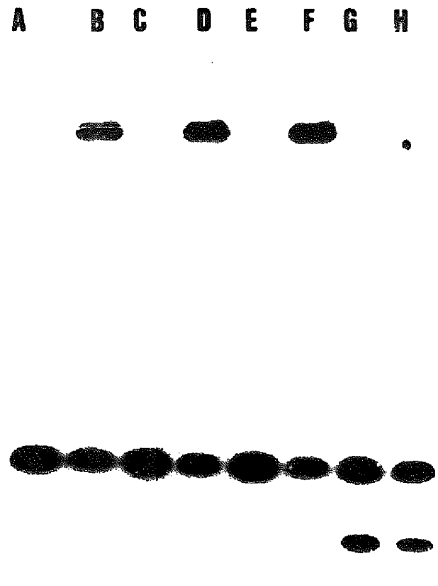

Fig. 2. Autoradiogram showing oligomerisation of α-toxin on RRBC membranes. A 10% suspension of RRBC in Tris-buffered saline (TBS, pH 7) was mixed with ^{3}H-toxin (final concentration 8 μg/ml) and incubated at 0°C. The cells were washed free of radioactivity and lysed in cold 5 mM phosphate buffer, pH 8 [31]. The membrane proteins were subjected to SDS-PAGE autoradiography as for Fig. 1. The film exposure time was 6 weeks. Lanes A to F: ^{3}H-toxin/RRBC. A and B: 5 min at 0°C; C and D: 15 min at 0°C; E and F: 15 min at 0°C + 5 min at 37°C; Lanes G and H: ^{3}H-toxin without RRBC. Samples in lanes B, D, F, and H were not boiled before gel electrophoresis. Note: monomers in all lanes; hexamers in lanes B, D and F.

Corresponding experiments using rabbit red blood cells (RRBC) as target showed essentially similar results (Fig. 2). This finding was confirmed with lower amounts of non-radiolabelled toxin and using Western blotting with a monoclonal antibody (MAb) for its detection. Hexamers were shown to form on RRBC already after 5 min at 0°C with as little as 0.5 μg/ml of α-toxin (Fig.

Fig. 3. Western blot showing oligomerisation of native α-toxin on RRBC membranes. RRBC (10%) were treated with 5 and 0.5 μg/ml of α-toxin in TBS for 5 or 15 min at 0°C and processed as described for Fig. 2. The membrane bound toxin was detected by immunoblotting using MAb 12E as described in Materials and Methods. Lanes A and B: toxin without RRBC; lanes C and D: membrane proteins from RRBC not treated with toxin; E to N proteins from RRBC treated with toxin, 0.5 μg/ml (E, F, G, H) or 5 μg/ml (K, L, M, N) for 5 min (E, F and K, L) or 15 min (G, H and M, N). Samples in lanes B, D, F, H, L, N were not boiled before the SDS-PAGE. Note that the toxin concentration used here was much lower than in Fig. 2 and that the MAb did not detect any monomeric toxin in membrane samples prepared from toxin-treated RRBC. Hexamers are seen in lanes F, H, L and N.

3). No hemolysis was detectable at these conditions (data not shown). Note that this MAb did not detect any monomeric toxin in membrane samples prepared from toxin-treated RRBC (Fig. 3, lanes E to N).

Hexamers were also directly visualised for the first time by electron microscopy on membranes isolated from toxin-treated intact Y1 cells (Fig. 4). Likewise the toxin was found oligomerised at low temperature when

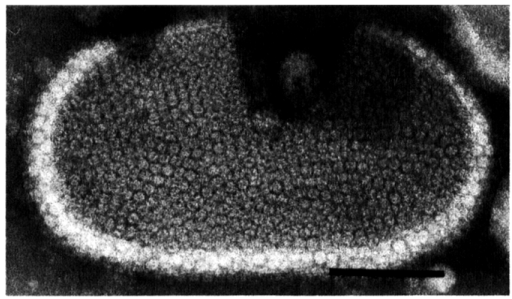

Fig. 4. Electron micrograph showing α-toxin oligomers on Y1 cells. Y1-cells were incubated with α-toxin (36 μg/ml) for 15 min at 0°C followed by toxin-free growth medium for 45 min at 37°C. Samples of isolated membranes were negatively stained with 1% Na-PTA (pH 7.1). Bar 100 nm.

added to intact platelets (Fig. 5) or to liposomes prepared of lipid extracts from platelet membranes (Fig. 5B).

In conclusion, at low temperature when α-toxin does not induce any membrane permeabilisation it forms oligomers on RRBC, platelet and Y1 cell membranes as well as on liposomes.

Oligomerisation of α-toxin on Y1 cells treated with concanavalin A

We have reported that pretreatment of Y1 cells with Con A inhibits the membrane damaging effect of subsequently added α-toxin [22]. It was now possible to clarify at which step Con A interferes with α-toxin action on Y1 cells; the binding, the putative conformational change or any possible later step necessary for induction of membrane permeabilisation.

We first ascertained that Con A prevented membrane permeabilisation of Y1 cells by high concentrations of α-toxin. Cells were pretreated for 20 min at

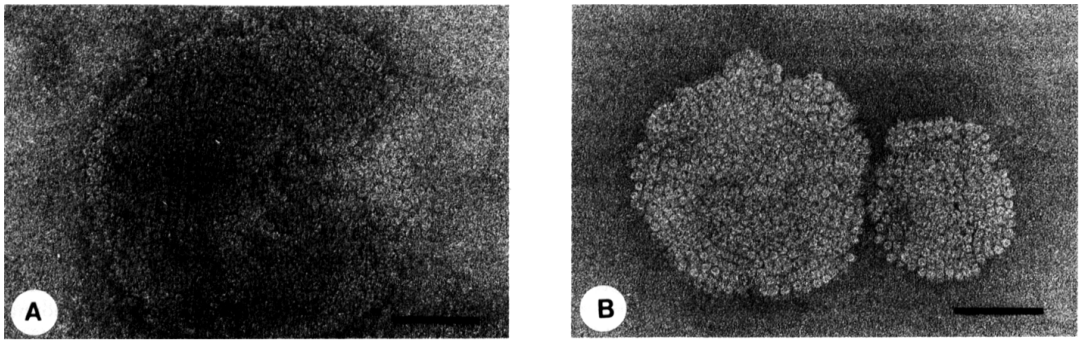

Fig. 5. Electron micrographs showing α-toxin oligomers formed at 2°C on intact platelets (A) and on liposomes prepared of lipid extracted from platelets (B). The platelets or extracted lipids (1 mg/ml) were mixed with α-toxin (5 μg) for 24 h at 2°C and subsequently stained with a mixture of PTA-glucose (0.5%/0.5%) for 1 min. Bars 100 nm.

22°C with the lectin and then incubated at 37°C with up to 50 μg/ml of the toxin. Con A at concentrations from 500 to 25 μg/ml afforded complete protection against membrane damage (data not shown). Thus Con A was used at 25 μg/ml in subsequent oligomerisation experiments. These showed that Con A blocked neither the binding of α-toxin at 0°C, nor toxin oligomerisation, which indeed took place already at low temperature whether Con A was present or not (Fig. 6).

It can be concluded that Con A present on the Y1 cell membrane interferes with the membrane damage caused by α-toxin in a step not related to the toxin oligomerisation.

Similar experiments were attempted with RRBC as target but these cells readily agglutinated during the preincubation with Con A, since in contrast to Y1 cells the RRBC were in suspension. Subsequently added α-toxin did not lyse the RRBC. However, a large part of the cells were probably not adequately accessible to the toxin because of the hemagglutination. For this reason toxin oligomerisation on RRBC pretreated with Con A was not studied.

Oligomerisation of α-toxin on Y1 cells and platelets in the presence of calcium

Addition of $CaCl_2$ to Y1 cells bearing membrane-bound toxin was previously shown to prevent membrane permeabilisation if the Ca^{2+} was added before the temperature was elevated. By contrast the presence of $CaCl_2$ only during the toxin binding step did not protect against intoxication [12]. It was hypothesised that Ca^{2+} inhibited the putative post-binding conformational change of the toxin, which was thought to consist in its oligomerisation. The influence of Ca^{2+} on oligomerisation was now tested directly by addition of $CaCl_2$ together with the toxin, or directly after the toxin-binding step. Oligomers of unchanged molecular size were detected whether the extra Ca^{2+} was totally omitted or it was added with the toxin or after the toxin binding step (Fig. 7). Similar results were obtained on liposomes by electron microscopy (Fig. 8). In conclusion, Ca^{2+} did not influence the oligomerisation process at all; thus it apparently affects an event taking place after toxin oligomerisation.

Influence of antibodies on toxin-induced membrane damage and on membrane-bound toxin

Polyclonal antibodies against α-toxin were previously shown to neutralise the toxin effect completely when added to Y1 cells directly after the toxin binding step [12]. Thus we now investigated whether antibodies would somehow affect the toxin oligomers.

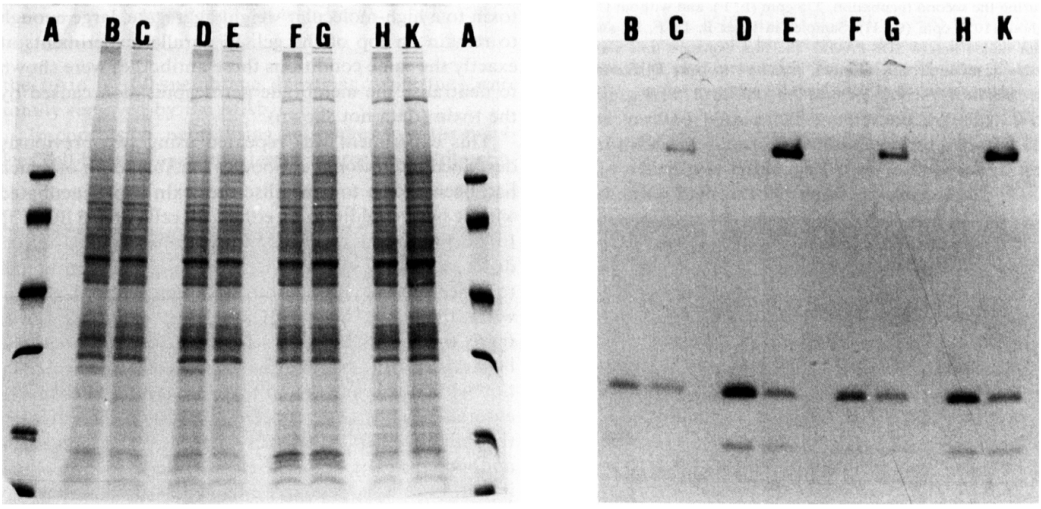

Fig. 6. SDS-gel and corresponding autoradiogram showing oligomerisation of α-toxin on Con A-treated Y1 cells. (A) SDS-gel stained with Coomassie blue. Lane A: molecular weight markers. From top: phosphorylase *b*, 94 K; albumin, 67 K; ovalbumin, 43 K; carbonic anhydrase, 30 K; trypsin inhibitor, 20 K, α-lactalbumin, 14.4 K. (B) Autoradiogram. The film exposure time was 10 weeks. ^{3}H-toxin (4 μg/ml) was incubated with Y1 cells which had been pretreated with Con A (25 μg/ml, 20 min, 22°C). Lanes B to E: Con A-treatment; B and C: toxin for 15 min at 0°C; D and E: toxin for 15 min at 0°C followed by toxin-free medium for 45 min at 37°C. Lanes F to K: controls without Con A; F and G toxin for 15 min at 0°C; H and K: toxin for 15 min at 0°C followed by toxin-free medium for 45 min at 37°C. The samples in lanes C, E, G, K were not boiled before SDS-PAGE. The absolute amounts of radioactivity in the solutions of membrane proteins (cpm/10 μl) were: 576 and 998 for the Con A-pretreated cells, and 642 and 953 for the controls without Con A after 15 min and 15+45 min, respectively. Note: monomers in all lanes, hexamers in lanes C, E, G and K.

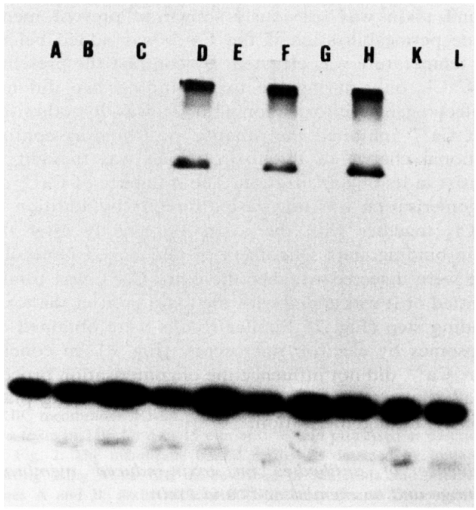

Fig. 7. Autoradiogram showing oligomerisation of α-toxin on Y1 cells in the presence of $CaCl_2$. Y1 cells were incubated in the presence or absence of $CaCl_2$ (50 mM) with 16 μg/ml ^{3}H-toxin for 15 min at 0 °C. Following two rinses the cells were further incubated for 45 min at 37 °C with or without $CaCl_2$. ^{3}H-toxin with Ca^{2+} (A and B) and without Ca^{2+} (K and L); lanes C to H: ^{3}H-toxin/Y1 membranes with Ca^{2+} throughout both incubations, 629 cpm (C, D), with Ca^{2+} only during the second incubation, 775 cpm (E, F), and without Ca^{2+} throughout, 1057 cpm (G, H). Samples in lanes B, D, F, H, and L were not boiled before SDS-PAGE. The film exposure time was 10 weeks. Note: monomers in all lanes, hexamers in lanes D, F and H, aggregate on top of same lanes for unknown reason.

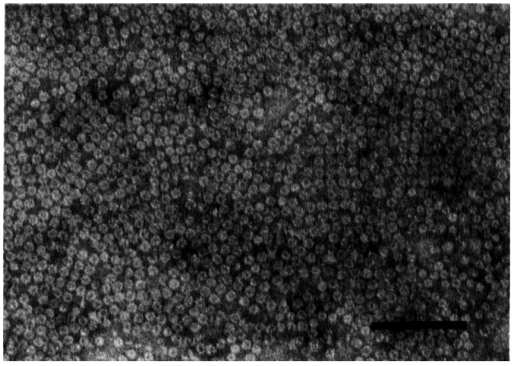

Fig. 8. Electron micrograph showing α-toxin oligomers formed on liposomes in the presence of 5 mM $CaCl_2$. α-Toxin (5 μg) was mixed with a lipid suspension (0.2 mg/ml) in TBS containing 5 mM $CaCl_2$ for 1 h at 3 °C. In order to remove debris the samples were centrifuged for 5 min at 1100 × g, 2 °C, and subsequently stained with 1% PTA (pH 7.0). Bar 100 nm.

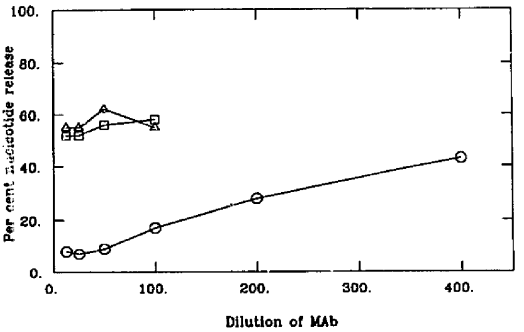

Fig. 9. Influence of monoclonal antibodies on plasma membrane damage induced by cellbound α-toxin. [^{3}H]Nucleotide-labelled Y1 cells were treated with 2 μg/ml α-toxin for 15 min at 0 °C, whereafter the cells were rinsed twice with ice-cold HBSS. Ice-cold solutions of ascites fluid containing MAb 12E (○——○) or 10D (△——△) diluted in growth medium were added, and after 5 min at 0 °C the cultures were transferred to 37 °C and further incubated for 24 h. The ^{3}H release is expressed as percentage of the maximal release obtained after complete rupture of the plasma membranes with 0.06 M sodium borate buffer (pH 7.8). ^{3}H release from control cells treated with toxin but no antibody was 57%. (□——□) O-ascites added instead of MAb.

It was found that polyclonal antibodies added directly after the toxin-binding step collected all of the toxin to a high-molecular weight aggregate, large enough to remain on top of the gel. In parallel experiments at exactly the same conditions these antibodies were shown to neutralise the membrane permeabilisation caused by the toxin (data not shown).

This experiment was repeated using two previously described monoclonal antibodies (MAbs), both of which had been shown to neutralise the toxin if pre-incubated with it before addition to either Y1 cells or RRBC [15]. Interestingly we now found that one of the MAbs, denoted as 12E, was able to neutralise the toxin bound to Y1 cells upon addition directly after the binding step, while the other, MAb 10D, did not neutralise under these conditions (Fig. 9). This differential functional behaviour of the two MAbs was reflected in their reaction with membrane bound toxin as seen on SDS-PAGE autoradiography (Fig. 10). Upon addition directly after the binding step MAb 12E acted as the polyclonal antibodies, i.e., it removed all hexameric toxin, collecting it into a large aggregate (Fig. 10, lane D). By contrast cells treated with the MAb 10D yielded membranes which contained a similar amount of hexamer as control membranes which had not been in contact with antibody (Fig. 10, lane F). For unknown reasons, in this experiment the toxin aggregated to a certain extent also in the control membranes and the MAb 10D-treated ones. However, this does not change the fact that there remained hexamers at both these conditions at which

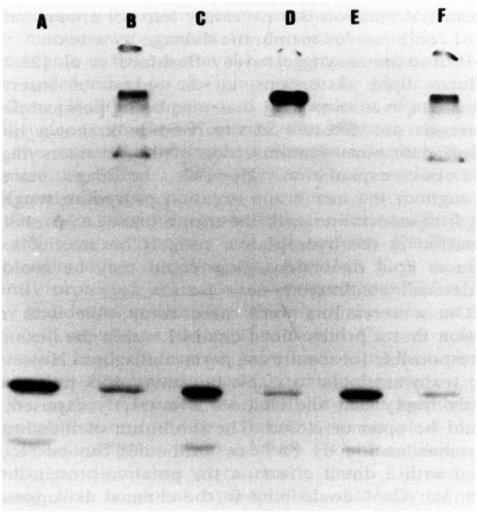

Fig. 10. Autoradiogram showing α-toxin oligomerisation in presence of monoclonal antibodies. Y1 cells were treated with 4 μg ^{3}H-toxin and 1/12.5 dilutions of MAbs as described for Fig. 9. Lanes A, B: toxin and no MAb; lanes C, D: toxin followed by MAb 12E; lanes E, F: toxin followed by MAb 10D. Samples in lanes B, D, F were not boiled before SDS-PAGE. Note: monomers in all lanes, hexamers in lanes B and F but not in lane D.

the toxin is still membrane damaging while they were totally removed by the MAb 12E.

In conclusion, neutralising antibodies to α-toxin collect cell-bound toxin into larger aggregates, while non-neutralising antibodies do not change the amount of hexamer in the membranes of Y1-cells.

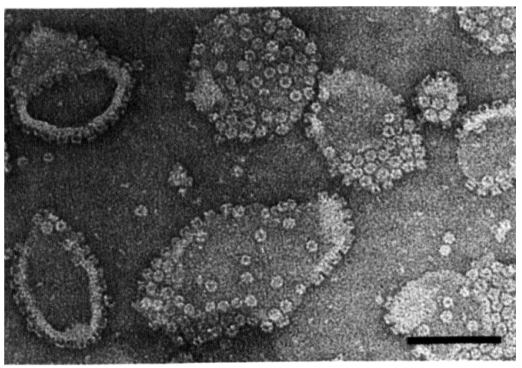

Fig. 11. Electron micrograph showing oligomers formed by ^{3}H-labelled tryptic fragments of α-toxin on preformed lipid layers. The lipid layer was produced by adding a lipid suspension on a water surface and lifting a carbon coated grid through the air/water interface. 2.5 μg of trypsin-treated α-toxin in 5 μl TBS was placed on the grid for 1 min at 22°C and stained with 1% PTA (pH 7.0). Bar 100 nm.

Oligomerisation of trypsin-treated α-toxin

Previous experiments showed that also fragments of α-toxin, despite their lack of membrane damaging activity, were able to form oligomeric approx. 200 kDa aggregates on Y1 cells [13]. As these oligomers were detected only by biochemical methods we now investigated by electron microscopy how they compared with the oligomers formed by intact α-toxin. The oligomers formed on liposomes from a solution of tryptic fragments (Fig. 11) resemble those formed from intact α-toxin (Fig. 5B). However, a closer inspection of these oligomers by computerised image analysis indicates subtle differences, e.g., a somewhat less compact structure than in the oligomers formed from intact toxin (to be published separately).

Discussion

As hexamers of α-toxin form on Y1 cells at quite unexpected conditions one can ask whether the hexamers in some cases could be artefacts arising during membrane isolation. That membrane-bound α-toxin forms hexamers on red blood cells, liposomes and Y1 cells has been established previously with the same methods as used in this work [1,3,13] and it was not discussed whether they could be artefacts. Indeed this problem is not easily circumvented. To ensure that we were not dealing with artefacts in the Y1 cell system the results were verified with other membrane systems. We also used different detection techniques and exposed cells to toxin which was either native or radiolabelled, and we used either high or low toxin concentrations.

Preparation of membranes from red blood cells involves somewhat less manipulation than with Y1 cells but the results were the same with both kinds of cells. In addition the EM-technique was adopted because as applied here it involved even less membrane manipulation. It was clear also with this technique that oligomers form on membranes rapidly regardless of temperature. The toxin concentrations used for preparation of electron micrographs were relatively high but the same results were obtained with low toxin concentrations on RRBC. Another advantage was the use of Western blot in parallel with autoradiography for detection of the toxin. Radiolabelling *could* have changed the toxin to act in some way anomalously compared to the native toxin used in the Western blot system, but apparently this was not the case.

The SDS-PAGE technique is highly reproducible and the oligomers are invariably hexamers as far as can be determined from their R_f values compared to those of marker molecules (a minor change of molecular size as

caused by, e.g., addition of Ca^{2+} would go undetected in SDS-PAGE). Thus it is clear that the hexamer represents the biochemically most stable form of the α-toxin oligomer at a variety of conditions. In conclusion, within the limits of what appears currently possible, we find it justified to assume that neither we nor previous authors have been dealing with an artefact.

The induction of membrane damage by α-toxin has been thought to take place in a series of separable events: (i) binding of toxin monomers to the membrane at low temperature, (ii) toxin oligomerisation on or in the membrane upon warming, and (iii) membrane permeabilisation. There is general consensus concerning the first two steps. However, three possible alternatives are currently discussed as regards the mechanism for the final membrane permeabilising event. First, the toxin oligomer could form within itself a hydrophilic channel through which materials can leak in and out [1]. Second, oligomers when inserted in the membrane could give rise to lipid distorsions along their edges allowing leakage in and out [23,24]. Third, the monomer could be the active toxin species, oligomers being irrelevant side products [2,4].

The concept that oligomeric protein-lined channels are responsible for membrane permeabilisation originated from a series of investigations carried out by Bhakdi and collaborators [1,3,25]. In a recent work from this group lysis of RRBC was shown to be accompanied by toxin oligomerisation [6] but it was not discussed whether also oligomerisation is always accompanied by hemolysis. In fact, a close examination of that work indicates the presence of oligomer after 60 min toxin exposure at 0 °C while there is no hemolysis at all even after 90 min at 0 °C with the same amount of toxin (see Ref. 6, Figs. 1B and 2).

Bashford and collaborators suggested that the membrane injury caused by α-toxin in cultured cells might occur at the edges of oligomers inserted in the membranes [23,24]. It was recently reported that at least in artificial lipid membranes no injury was detectable at conditions when only monomers were present in the membranes [5]. The data suggest that the presence of oligomers containing at least four monomers is a prerequisite for channel formation and the results are compatible with the notion that the oligomers contain protein-lined channels.

In the present work we have tested the two hypotheses that: (i) membrane permeabilisation is always accompanied by hexamer formation and (ii) hexamer formation always leads to membrane permeabilisation. Without exception our data indicate the presence of hexamers when membrane permeabilisation occurs. However, hexamers are detected also at conditions when no membrane permeabilisation is detectable. Thus our data are consistent with hypothesis (i) while hypothesis (ii) is refuted. A major conclusion is therefore that hexamer formation is a necessary but not always sufficient condition for membrane damage by α-toxin.

If α-toxin, as suggested by Bashford et al. [23,24], induces lipid distorsions at the edges of inserted hexamers, it is surprising that membrane permeabilisation was not detected at the conditions shown here. Insufficient membrane insertion of the hexamers might give one explanation. However, hexamers remain throughout the membrane isolation procedure suggesting firm association with the membranes, i.e., probably insertion in the hydrophobic core. If hexameric toxin induces lipid distorsions, how could they be avoided under such conditions?

Our observations were more compatible with the notion that a protein-lined channel within the hexamer is responsible for membrane permeabilisation. However, the toxin assembly to stable hexamers does not necessarily imply that the channel is correctly exposed; it could be open or closed. The inhibition of membrane permeabilisation by Ca^{2+} or antibodies can be reconciled with a direct effect on the putative protein-lined channel: Ca^{2+} could bind in the channel as suggested previously [8]. Antibodies are seen to collect all of the membrane-bound hexamers to larger aggregates, implying removal of the channels from their right transmembrane position.

The inhibition of membrane damage at low temperature in spite of oligomer formation would be fully consistent with the lipid distortion theory, but also the putative channel might be affected by low temperature. We have recently determined the three-dimensional (3D-)structure of α-toxin oligomers on lipid layers [9]. They have a 'lid' covering the entrance to a cavity and also appear to penetrate only a short distance into the membrane. Hexamers formed at physiological temperature have not yet been investigated with respect to 3D-structure because of difficulties to obtain useful crystalline layers. However, projection structures of such oligomers have more stain in the center of the oligomer [9,26] and thus appear to have an open channel in contrast to the oligomers formed at low temperature. Likewise recent computerized image analyses of oligomers formed from tryptic fragments suggest that also these lack functional transmembrane channels despite the assembly to ring-like structures (Fig. 5) closely resembling those formed from intact α-toxin (to be published separately).

The tetragonal crystals used for the 3D-reconstruction occur more frequently when using lipid layers instead of intact membranes. The working hypothesis is then that this 3D-structure of the native toxin represents an initial binding conformation, and that a structural transition is necessary for obtaining the final membrane damaging form. It is possible to interpret the 3D-map as consisting of monomers forming a 'V' with a 'hinge region' binding to the membrane. This is in

accordance with spectroscopic measurements indicating the monomer to consist of two domains [27]. It is reasonable to believe that the conformational transition rate is dependent on the temperature. In addition some authors [28,29] have proposed the existence of a specific cell surface receptor for α-toxin. The efficient triggering of the transition by such a specific receptor might explain the different sensitivities to α-toxin exhibited by different cell types.

However, previous proposals that α-toxin might bind to the Con A receptor on cell surfaces [29,30] are contradicted by our findings. Although Con A efficiently protected against toxin-induced membrane injury it did not prevent firm binding of the toxin to the Y1 cell membrane. Membrane-bound Con A might sterically hinder a correct insertion of the hexameric channel or alternatively mask the channel somehow.

We earlier discussed the possibility that membrane permeabilisation could be induced by *monomeric* α-toxin [2]. The same point was raised by Harshman and collaborators discussing more recent data: "it can be argued that a global hydrophobic character of the monomer ...facilitates assembly into hexamer form. Alternatively ...the global hydrophobic character of the monomer... facilitates a focal distorsion of the lipid bilayer... These two possible models cannot be resolved by the data at hand." [4]. Interestingly, the present work resolves this question thanks to the unique properties of the MAb 12E. With high sensitivity this antibody detects native monomeric α-toxin as well as toxin oligomers (Fig. 3 and data not shown). However, it is also clear from Western blots that the MAb 12E does not detect monomeric toxin generated from membrane-bound toxin by boiling in SDS (Fig. 3). Neither is any monomeric toxin seen along with the oligomeric form in the lanes containing unboiled samples. This contrasts all previous studies in which either autoradiography (Ref. 13 and this work) or Western blot with polyclonal antibodies [3,25] indicated the presence of monomers along with oligomeric toxin. These monomers are seen both at membrane damaging conditions and at conditions when the membrane damage had been prevented (Figs. 1, 2, 6, 7 and 10). Thus, they appear of no relevance for the membrane permeabilising event and they most probably represent toxin which has been dissociated from the oligomeric form, a possibility already pointed out [3]. The hypothesis that the monomeric form could be the membrane active species of α-toxin is therefore contradicted.

In conclusion, α-toxin upon membrane contact rapidly and independently of temperature assembles to hexameric aggregates. The toxin appears completely converted to the oligomeric form, suggesting that the active toxin species cannot be monomeric. Indeed hexamers are always detected along with membrane permeabilisation. However, hexamers are also detected at various conditions when membrane permeabilisation is prevented. Apparently the hexamer can exist in different conformations, not all of which are functional. We suggest that hexamers which do not induce membrane injury do not correctly expose the internal hydrophilic channel which is necessary for membrane permeabilisation.

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