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Release of lipid vesicle contents by the bacterial protein toxin α-haemolysin

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α-Haemolysin is a protein toxin (107 kDa) secreted by some pathogenic strains of *E. coli*. It binds to mammalian cell membranes, disrupting cellular activities and lysing cells. This paper describes the mechanism of α-haemolysin-induced membrane leakage, from experiments in which extrusion large unilamellar vesicles, loaded with fluorescent solutes, are treated with purified toxin. The results show that the toxin does not require of any membrane receptor to exert its activity, that vesicles become leaky following an 'all-or-none' mechanism, and that leakage occurs through a non-osmotic detergent-like bilayer disruption induced by the protein. Small pores formed by monomeric α-haemolysin, as described by other authors, do not appear to be related to the process of membrane disruption. Instead, the experimental data would be in agreement with the idea of oligomeric assemblies being required to produce release of solutes from a single vesicle.

Introduction

α-Haemolysin is a protein (107 kDa) which is actively secreted by *E. coli*. It appears to be responsible for the pathogenic effects of some strains of the bacterium, and it has also the added interest of being one of the very few proteins for which *E. coli* has a specific export machinery [1]. As a toxin, α-haemolysin binds to mammalian cell membranes, disrupting cellular activities and lysing cells. Jorgensen et al. [2] were among the first to study the membrane effects of this toxin. Bhakdi et al. [3] examined the toxin-induced release of ions and haemoglobin from red blood cells, and proposed that α-haemolysin might damage target cell membranes by generating monomeric transmembrane pores, 1.5–3 nm in diameter, a view that was supported by studies using model membranes [4,5].

From the point of view of structure, the C-terminal region of α-haemolysin contains a 9-amino-acid chain that is reiterated 11 times within a 129-residue span [1]; alternatively, a 13-fold repetition of an octapeptide has been suggested [6]. Alterations of these amino-acid repeats affect cytolytic activity and secretion [6]. More recently, it has been shown that the mature toxin contains one or more fatty acyl residues [7]. This, together with the stretches of non-polar amino-acids that are found near the N-terminal end [8,9] explain the amphipathic behaviour of the protein, which can be found in an active form both in polar and non-polar environments. The partly hydrophobic character of α-haemolysin also explains that, in aqueous media, it is protected against denaturation by 6 M urea [10] and, also in aqueous environments, it is found in the form of large aggregates [11].

The present contribution is intended to describe in detail the phenomenon of α-haemolysin-induced membrane leakage, by using large unilamellar vesicles, obtained by extrusion, as a membrane model. In particular it will be shown how leakage does not appear to occur through discrete pores, but is rather the result of a detergent-like effect of the toxin, which disrupts the lipid membrane, probably by facilitating the formation of non-bilayer structures.

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Abbreviations: ANTS, 1,3,6-trisulphonate-8-aminonaphthalene; DPX, *p*-xylenebis(pyridinium bromide); PC, phosphatidylcholine; PE, phosphatidylethanolamine; CHOL, cholesterol; FITC, fluorescein isothiocyanate; LUV, large unilamellar vesicles; HU, haemolytic unit.

Materials and Methods

Materials

Egg phosphatidylcholine (PC), and egg phosphatidylethanolamine (PE), were grade I from Lipid Products (South Nutfield, England). Cholesterol (CHOL) and all other lipids were from Sigma (St. Louis, MO). 8-Aminonaphthalene-1,2,3-trisulphonic acid (ANTS), *p*-xylylenebispyridinium bromide (DPX) and octadecylrhodamine B (R_{18}) were obtained from Molecular Probes (Eugene, OR). Dextrans and fluoresceine isothiocyanate (FITC)-derivatised dextrans were also purchased from Sigma. α-Haemolysin was purified from the culture filtrates of an over-producing strain of *E. coli*, according to Ostolaza et al. [11]; the haemolytic activity of the preparations was of about 400 haemolytic units (HU)/μg protein ($\approx$ 43 000 HU/nmol), 1 HU being defined as the dilution factor required for a given α-haemolysin preparation to produce 50% lysis of a standard red blood cell suspension [12].

Vesicle preparation and measurements of leakage

Large unilamellar vesicles (LUV) of different compositions were prepared by extrusion and sized using 0.1 μm pore size Nuclepore membranes as described by Mayer et al. [13]. Vesicle contents leakage was measured by using the ANTS/DPX system according to Ellens et al. [14]. Liposomes contained 12.5 mM ANTS, 45 mM DPX, 50 mM Tris-HCl (pH 7.0). Liposomes were separated from unencapsulated material by column chromatography on Sephadex G-75 using 50 mM Tris-HCl, 85 mM NaCl, 10 mM $CaCl_2$ (pH 7.0) as the elution buffer. The same buffer was used in the leakage assays. When required, strictly isotonic conditions were ensured by checking the osmotic pressure of intra- and extravesicular solutions in an Osmomat 30 osmometer (Gonotec, Berlin).

Fluorescence assays were performed in a LS-50 Perkin Elmer spectrofluorometer, at room temperature (20–22°C) with continuosly stirred cuvettes. For the ANTS/DPX system, excitation was at 355 nm and emission at 530 nm. An interference filter (Schott 06505, 515 nm) was used to avoid scattered light. 100 μl of a liposome suspension ($5 \cdot 10^{-5}$ M in lipid) were diluted in 400 μl buffer; the fluorescence level of this preparation did not vary with time, and was set as 0% leakage. The 100% fluorescence level for leakage was obtained by detergent lysis (Triton X-100, 4 mM final concentration) of the liposomes. Leakage experiments were started by adding an appropriate amount of purified α-haemolysin (usually 400–600 HU in about 20 μl) to the intact liposome suspension. (Crude toxin preparations were not suitable for these assays, since they contained highly fluorescent impurities.) Corrections for differences in the amount of solutes entrapped in the various vesicle preparations were carried out when required [15]. Since α-haemolysin is kept in 6 M urea for stability reasons [11] it was checked that urea, up to 0.5 M in the cuvette, did not interfere with our measurements; in practice, urea concentration in the cuvette was always below 0.25 M.

In some experiments, the release of fluorescein isothiocyanate (FTIC), or FITC-derivatised dextrans, was tested. The self-quenching properties of FITC were used in this series of measurements. Liposomes prepared in 4.36 mM FITC, or FITC-dextrans, were freed from non-encapsulated material using a column of Sephacryl S-300 (30 $\times$ 2.5 cm), eluted with 50 mM Tris, 70 mM NaCl, 0.1 mM EDTA (pH 7.0). Fluorometry conditions were: excitation, 465 nm; emission, 520 nm; interference filter, 495 nm.

Lipid mixing

Vesicle lipid mixing was measured by dilution in the bilayer of the self-quenching probe octadecylrhodamine (R_{18}) as described by Hoekstra et al. [16]. The details were as described previously [15].

Leakage mechanism

A series of measurements was carried out to find out whether partial leakage occurs as a result of an all-or-none event (some of the vesicles release all of their contents) or as a graded event (all of the vesicles release part of their contents), essentially as described by Parente et al. [17]. Briefly, a quench curve for vesicles containing varying concentrations of ANTS and DPX, always at a 3.6/1 mole ratio, was constructed. Then the extent of leakage induced by α-haemolysin was measured at a variety of lipid/protein ratios, keeping constant lipid concentration. Samples were applied to a Sephadex G-75 column to separate the lipid vesicles; fluorescence of the eluted vesicle fraction was measured before and after detergent solubilization, and the percent fluorescence remaining with the vesicles calculated. Experimental values can be compared with predicted results [17,18].

Results

When LUV are treated with α-haemolysin, release of vesicle contents occurs (Fig. 1A) until a stable fluorescence value is obtained (in the example shown, in about 15 min). In the curve in Fig. 1A, equilibrium is reached well before 100% leakage has occurred. This is very often the case under our experimental conditions, in fact 100% release of liposomal contents is not usually observed. This phenomenon will be examined in detail later. No lipid mixing is detected (Fig. 1B) under these or any other experimental conditions tested, suggesting that α-haemolysin does not induce vesicle fusion.

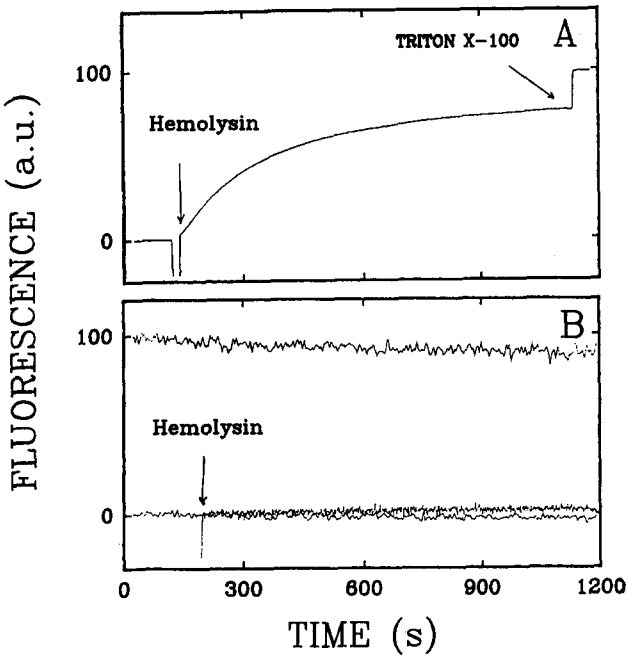

Fig. 1. Assays for α-haemolysin-dependent vesicle leakage and vesicle fusion. (A) Release of aqueous contents (ANTS/DPX) from LUV (PC:PE:CHOL, 2:1:1). Lipid and protein concentrations were 10 μM and 50 nM, respectively. 100% leakage was measured after addition of 4 mM Triton X-100. (B) Lipid mixing assay, based on dequenching of R_{18}. The lower, superimposed, curves correspond to experiments in the presence or absence of α-haemolysin. The upper trace was recorded in the presence of 4 mM Triton X-100, and corresponds to 100% fusion (lipid mixing).

In order to explore the influence of vesicle lipid composition on the phenomenon of leakage, experiments were carried out with different lipid mixtures. A comparison parameter was arbitrarily set as the extent of leakage produced 10 min after addition of 400 HU of α-haemolysin, a condition under which maximum release was never observed. (Similar results were found when the amount of α-haemolysin was varied between 40 and 800 HU.) The data are shown in Table I; it can be seen that changes in lipid composition may induce up to 4-fold changes in activity. It should also be noted that, although the total lipid extract from red blood cells supports a high leakage activity when compared to pure PC, some very simple formulations are even more sensitive to α-haemolysin. Analysis of the data in Table I also reveals that some lipids, such as PE and CHOL, greatly increase the observed leakage, while others are not so effective. It is also noteworthy that lysolecithin appears to reverse the effect of PE, so that the PC:PE:lysolecithin (2:1:1) mixture is actually less effective in promoting leakage than pure PC. In view of the high leakage activity observed with PC:PE:CHOL (2:1:1) all further experiments were carried out with this mixture, unless otherwise specified.

The kinetics of the leakage process was studied by performing release experiments, as shown in Fig. 1,

TABLE I

The influence of bilayer lipid composition on α-haemolysin-induced release of vesicle contents

Lipid composition (mole ratio)	% Release
PC	27
PC:PE (2:1)	67
PC:CHOL (3:1)	69
PC:PE:CHOL (2:1:1)	76
PC:PE:lysolecithin (2:1:1)	20
PC:PI (1:1)	42
PC:sphingomyelin	40
PC:CHOL:sphingomyelin:PE:cerebroside (6:5:4:4:1)	57
Red blood cell lipids	61
'Outer monolayer' lipids [b]	69

[a] % Release measured 10 min after addition of 400 HU (≈ 1 μg) of α-haemolysin. Lipid concentration was 10 μM.

[b] An artificial mixture of lipids believed to represent the composition of the erythrocyte plasma membrane outer monolayer: PC:PE:CHOL:SM:ganglioside (12:3:17:10:1).

with varying amounts of α-haemolysin, while keeping constant lipid concentration (10 μM). At each protein concentration, a $t_{1/2}$ is measured, as time at which half-maximal release is produced. The resulting data are plotted as a function of toxin concentration, as shown in Fig. 2. A plot of $t_{1/2}$ vs. reciprocal of α-haemolysin concentration gives a straight line (inset), suggesting that the process is second order with respect to α-haemolysin. The reciprocal of the slope of this line gives an experimental velocity constant $k_{exp} = 3.15$

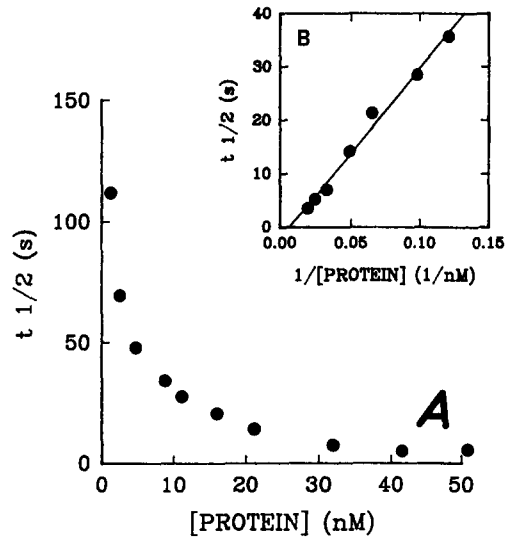

Fig. 2. Kinetics of α-haemolysin-induced vesicle leakage. Leakage experiments, as shown in Fig. 1, were performed with increasing amounts of toxin and $t_{1/2}$ (time required to produce a half-maximal effect) recorded for each case. (A) Direct plot of $t_{1/2}$ *vs.* α-haemolysin concentration. (B) Plot of $t_{1/2}$ *vs.* reciprocal of toxin concentration. The slope of the straight line is the reciprocal of the experimental rate constant k_{exp}. Vesicle composition was PC:PE:CHOL (2:1:1).

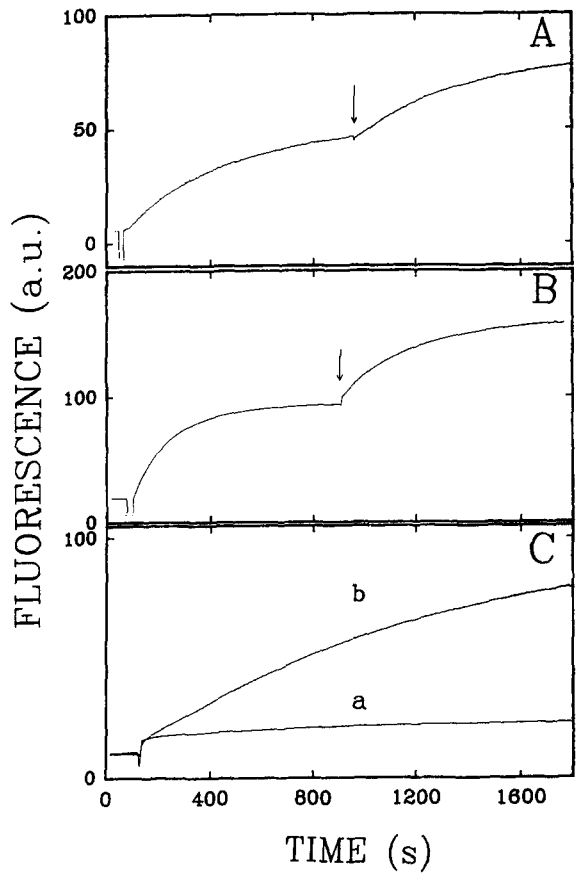

Fig. 3. Release of liposomal aqueous contents, induced by α-haemolysin. (A) A LUV preparation (10^{-4}M) is treated with an amount of toxin producing less than 50% release (1.5 μg); a second addition of the same amount of toxin (arrow) produces a similar release. (B) An experiment is started with the minimal amount of protein producing 100% release (4.5 μg); the addition of a fresh batch of liposomes (10^{-4} M); is again accompanied by release of contents. (C) Curve a: α-haemolysin (1.2 μg) is pre-incubated for 10 min with an excess (2 mM) of 'empty' LUV; then 0.2 mM LUV loaded with ANTS/DPX is added. Curve b: as in curve a, except that the protein was not pre-incubated with 'empty' vesicles.

$\cdot 10^{-3}$ $nM^{-1}s^{-1}$. Vesicles composed of some other lipid mixtures were analyzed in a similar way; second-order kinetic behaviour was found in all cases for a wide range of toxin concentrations. Measured k_{exp} values were: $1.56 \cdot 10^{-3}$ $nM^{-1}s^{-1}$ (pure PC), $1.98 \cdot 10^{-3}$ $nM^{-1}s^{-1}$ (PC:CHOL, 3:1), or $2.52 \cdot 10^{-3}$ $nM^{-1}s^{-1}$ (PC:sphingomyelin, 3:1).

When lipid vesicles are treated with an amount of toxin such that less than 50% leakage occurs, a second addition of the same amount of toxin produces again the same extent of leakage (Fig. 3A). In general, the final extent of leakage is the same irrespective of whether α-haemolysin has been added in one or more aliquots. The results in Fig. 3A, apart from showing the reproducibility of α-haemolysin action, appear to indicate that, at least under certain conditions, some vesicles are preserved from the toxin. This, in turn, may explain why, in some cases, 100% leakage is not reached in our experiments, and speaks in favour of an 'all-or-none' leakage mechanism (see below).

The reciprocal experiment, in which, once a plateau has been reached in a leakage experiment, a fresh, similar aliquot of vesicles is added, is shown in Fig. 3B. Again, release occurs until a plateau is reached, and the two segments of the curve are virtually superimposable. This experiment could be interpreted at least in two ways: (i) α-haemolysin comes in and out of the membrane, thus a fresh population of vesicles may be attacked just as well as the original one, or (ii) membrane binding may be irreversible, or only slowly reversible, but the membrane/water partition coefficient of α-haemolysin is rather low, so that, at this protein/lipid ratio, the bulk of the protein is in solution, ready to partition into new aliquots of fresh vesicles. However, the experiment shown in Fig. 3C strongly supports the latter hypothesis; in this experiment α-haemolysin (12 μg) was preincubated for 10 min with a large excess (2 mM) of 'empty', i.e., non-fluorescent, LUV. Then, at time zero, an aliquot (0.2 mM) of vesicles containing ANTS and DPX was added. Release (curve a) is negligible. Curve b is the positive control, in which no pre-incubation with empty liposomes had occurred. Thus the protein appears to bind irreversibly to the lipid bilayer, although under most experimental conditions a significant fraction of α-haemolysin remains free in solution.

Release of vesicle contents induced by α-haemolysin was further characterized in a series of experiments in which lipid and protein concentrations were independently varied (Fig. 4). Both the extent and the initial rates of leakage have been studied. The data in Fig. 4 correspond to pure egg PC vesicles, but similar results were found with PC:PE:CHOL (2:1:1). When lipid concentration is varied, at a constant protein/lipid mole ratio (1:2000) (Fig. 4A,B), the lytic activity increases almost linearly with concentration; this can also be interpreted as a result of the toxin partitioning between lipid and water: as the lipid concentration decreases, the fraction of membrane-bound toxin is also decreased. When α-haemolysin concentration was kept constant (0.1 μM) and lipid concentration was varied from 50 to 500 μM, only a slight decrease in the extent and initial rate of leakage was recorded (Fig. 4C,D). A decrease would in fact be expected according to our previous hypothesis of an irreversible toxin-bilayer binding; the fact that such a decrease is only moderate may reflect again that a significant fraction of the protein remains free in solution under most conditions. In a separate series of experiments, α-haemolysin concentration was varied, at constant lipid (10 μM) (Fig. 4E,F). Both percent leakage and initial rates increase very steeply with low toxin concentrations, finally reaching a plateau. The observed high

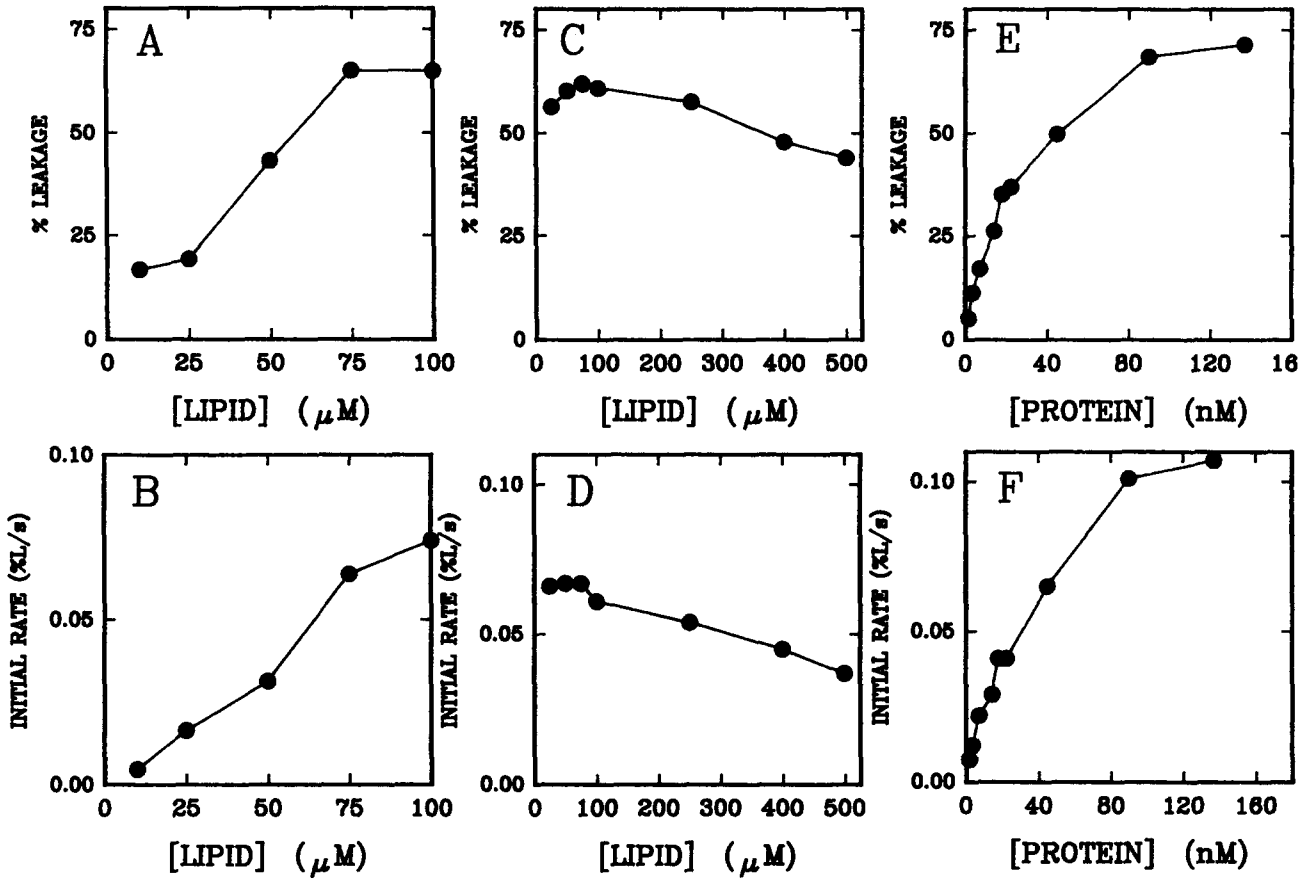

Fig. 4. Effect of independently varying lipid and protein concentrations on the extent and initial rate of vesicle leakage. (A,B) Lipid concentration is varied, at a constant (2000:1) lipid:protein mole ratio. (C,D) Lipid concentration is varied, at a constant (0.1 μM) protein concentration. (E,F) α-Haemolysin concentration is varied, at a constant (10 μM) lipid concentration. LUV were composed of PC.

sensitivity of leakage rates with respect to protein concentration suggests that a monomer-multimer assembly process may be involved in the leakage mechanism.

It has been mentioned that, under most experimental circumstances, leakage reaches a plateau at values below 100%. In principle, incomplete leakage may be due to either of two reasons, or to a combination of them, namely: (i) some, but not all, of the vesicles release all of their contents (all-or-none mechanism), or (ii) all of the vesicles release only part of their contents (graded mechanism). A method to ascertain whether or not one of these mechanisms is predominant was described by Parente et al. [17] and has been summarized above. Fig. 5 shows the results obtained with several protein/lipid ratios; the observed and predicted values (including those for either hypothesis) are shown. Comparison of those values clearly shows that release is best interpreted as an all-or-none mechanism, i.e., under conditions at which partial release is observed, there is a fraction of vesicles left intact after toxin treatment. This has important implications for the mechanism of action of α-haemolysin, as described below.

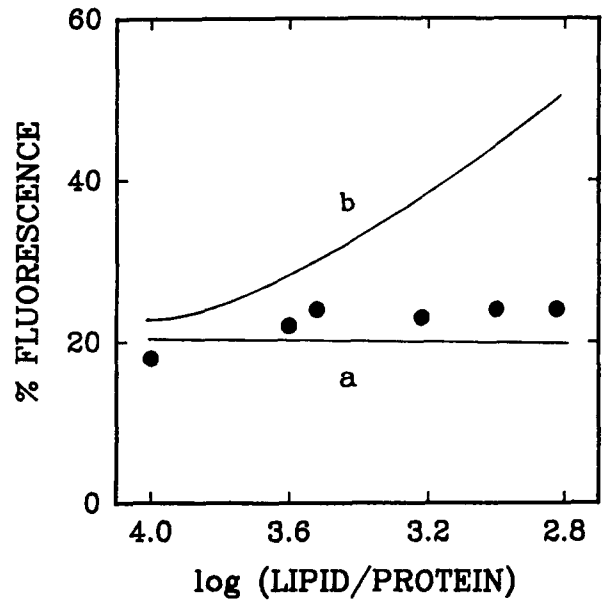

Fig. 5. An experimental test for the mechanism of α-haemolysin induced vesicle leakage. Curve a: theoretical values predicted for the 'all-or-none' mechanism. Curve b: theoretical values predicted by the 'gradual release' mechanism. Full circles: experimental values. (See text for details.)

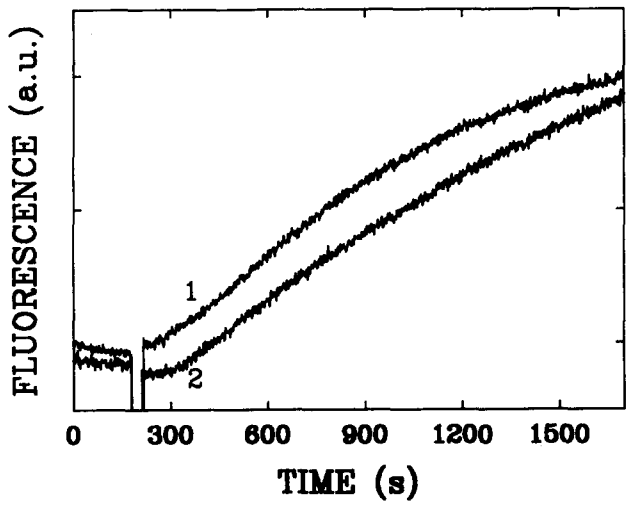

Fig. 6. α-Haemolysin-induced release of FITC and FITC-dextrans. Curve 1: release of FITC-dextran ($M_r = 17200$). Curve 2: experiment as in curve 1, except that non-fluorescent dextran had been added to the extravesicular buffer until the osmotic pressure exactly matched that of the vesicle contents.

All the above observations are compatible with the currently held view that α-haemolysin is a pore-forming toxin. However, it is also possible that the protein acts in a detergent-like way, disrupting the integrity of the bilayer. The following experiments appear in fact to support the latter hypothesis. Measurements were carried out of the leakage of vesicles loaded with fluorescent substances of increasing molecular weights. In particular, self-quenching FITC-derivatised dextrans were used. Fig. 6 shows the leakage of FITC-dextran, $M_r = 17200$. The fact that this large molecule may leave the vesicles speaks against a pore mechanism. It could be argued that haemolysin forms only small pores, through which small ions and water enter the vesicle until it bursts, thus liberating the entrapped FITC-dextran. However, dextran release does not appear either to be a secondary effect of an osmotic shock of the vesicles: curve 1 in Fig. 6 was obtained under the usual conditions, but curve 2 was recorded in a system in which non-fluorescent dextran in the same buffer was added to the outside of the vesicles until the outer osmotic pressure exactly matched the corresponding value inside the vesicles (osmotic pressures were measured with an osmometer, as indicated under Materials and Methods). No significant difference is observed between curves 1 and 2. It should be noted that dextran release curves appear rather sigmoidal in shape, as compared to that of small molecules, e.g., ANTS (Figs. 1 and 6). Finally, the results in Fig. 7 support the idea that dextrans are released via the same mechanism as the small molecules; all three FITC-dextrans display a similar behaviour in an experiment in which α-haemolysin concentration was changed (compare with Fig. 4E).

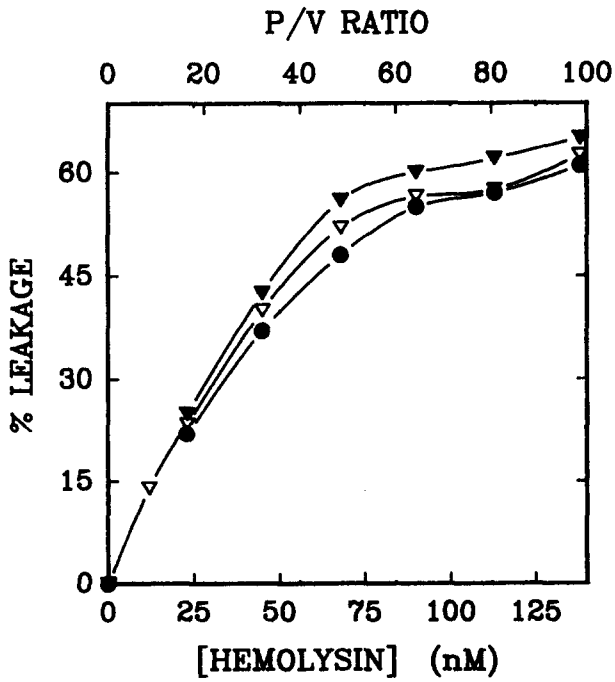

Fig. 7. α-Haemolysin-induced release of various FITC-dextrans. (●) $M_r = 17200$. (▼) $M_r = 9400$. (▽) $M_r = 4400$.

Discussion

The experimental results presented in this paper may provide answers to a number of questions, hitherto unclarified, on the mechanism of α-haemolysin-induced lysis of eukaryotic cells.

A receptor for α-haemolysin?

The existence of a receptor for α-haemolysin in the erythrocyte membrane had been postulated by several authors in the past. More recently, Benz et al. [19], studying toxin-dependent changes in bilayer conductivity, observed much higher toxin activity in membranes composed of asolectin than in those formed by pure phospholipids, PC or phosphatidylserine. These results were interpreted as suggesting a lipidic receptor, that would also exist in a complex mixture such as asolectin. However, our results in Table I clearly indicate that, although mixtures of natural origin, e.g. red blood cell lipids, support higher lytic activities than pure PC, simple lipid mixtures, e.g., PC:PE:CHOL, support similar if not higher activities. This does not necessarily exclude the presence of a receptor in cells, but explains all current observations without requiring its existence.

The leakage mechanism

Parente et al. [17] have distinguished between two putative leakage mechanisms, respectively the 'all-or-none' and the 'graded release' mechanisms. Their synthetic, amphipathic peptide GALA was found to follow

the former pattern. A recent report [20] has shown how synthetic surfactants may induce release from PC vesicles following one or the other of the above mechanisms, in different cases. Our results (Fig. 5) clearly suggest that α-haemolysin acts through the all-or-none mechanism. In turn, this implies that the number of toxin molecules required to induce complete leakage from a given vesicle must exceed a certain critical value [17]. When the number of vesicle-associated protein molecules is below that value, or perhaps when an appropriate number of bound molecules fails to reach a certain assembly pattern, leakage does not occur.

Pore formation vs. detergent-like effect

Several toxins are known to act via pore formation [21]. *E. coli* α-haemolysin has been included among them [5,19,31], and the pore diameter has been estimated at ≈ 1–3 nm. The main experimental evidence consists of electrophysiological studies in 'black lipid film' bilayers. Although the published data may support the existence of pores through which small molecules can permeate, it is evident that the non-osmotic leakage of high-molecular-weight dextrans described in this paper (Figs. 6,7) is not compatible with flux through such pores. Our studies do not rule out the possibility that, at some stage of bilayer-toxin interactions, the suggested small pores be formed, but they clearly demonstrate that an additional α-haemolysin effect is required to explain the observed leakage.

The simplest way to interpret our data is to assume that α-haemolysin-induced release of aqueous contents from large unilamellar vesicles is due to bilayer disruption in a detergent-like effect of the toxin. This mechanism of action has not been often invoked to explain the effect of bacterial toxins, but it is accepted in the case of other peptide toxins, e.g. melittin [22–25]. The bilayer-destabilizing properties of α-haemolysin are rather obvious from our data in Table I. Lipids such as PE and CHOL, which tend to destabilize the bilayer under certain conditions [26–29] allow the development of high leakage activities. Furthermore, lysolecithin, known to compensate the destabilizing effect of PE [30], also compensates the pro-lytic capacity of PE (Table I). We therefore suggest that leakage induced by α-haemolysin occurs through a toxin-induced bilayer disruption, perhaps through the formation of (transient) non-bilayer intermediates. The same mechanism could operate in cell as well as in model membranes.

A previous study on the effect of α-haemolysin on phospholipid vesicles [5] led to somewhat different conclusions, namely that the toxin makes the vesicles permeable (to calcein) by forming pores through the membrane. However, we believe that those and the present results are not directly comparable, and that the previously published data correspond to a different phenomenon induced by α-haemolysin. In the previous case, pseudo first-order kinetics was found, while we apparently find second-order. In addition, small unilamellar vesicles, prepared by sonication, instead of LUV, were used in that work; sonicated vesicles are metastable, and many protein and non-protein substances are known to induce their breakdown [32,33]. Differences in protein preparation might also account for the apparent disagreement, since Menestrina [5] appears to use only a partially purified α-haemolysin.

Monomer vs. multimer organization

Several reports have indicated that the aqueous channel formed by α-haemolysin consists of a single protein monomer [3–5]. More recently, however, the α-haemolysin channel has been suggested to be formed by a toxin oligomer [19]. As discussed above, it is possible that formation of voltage-dependent ion channels and vesicle leakage are two distinct, separate effects of α-haemolysin. The present work has been focused on the latter phenomenon, thus the pertinent question is whether a single toxin monomer is enough to break down the permeability barrier of LUV. Even though they are not conclusive in this respect, our results appear to favour the hypothesis of multimeric aggregates being responsible for the observed leakage. Significant observations in this respect are the second-order kinetics, suggesting that, at least at some stage in the process, the protein acts as a dimer (Fig. 2), the steep increase of the extent and rate of leakage upon increasing the protein/lipid ratio (Figs. 4E,F and 7) [17], and the all-or-none mechanism of leakage (Fig. 5) [17]. The ensemble of these data would be in agreement with the idea that the number of molecules required to produce release from a single vesicle must exceed a critical value; such a value could vary with the size of the solute to be released.

Acknowledgments

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References

1 Bhakdi, S., Mackman, N., Menestrina, G., Gray, L., Hugo, F., Saeger, W. and Holland, I.B. (1988) Eur. J. Epidemiol. 4, 135–143.
2 Jorgensen, S.Z., Hammer, R.F. and Wu, G.K. (1980) Infect. Immun. 27, 988–994.
3 Bhakdi, S., Mackman, N., Nicaud, J.M. and Holland, I.B. (1986) Infect. Immun. 52, 63–69.
4 Menestrina, G., Mackman, N., Holland, I.B. and Bhakdi, I.S. (1987) Biochim. Biophys. Acta 905, 109–117.
5 Menestrina, G. (1988) FEBS Lett. 232, 217–220.
6 Felmlee, T. and Welch, R.A. (1988) Proc. Natl. Acad. Sci. USA 85, 5269–5273.
7 Issartel, J.P., Koronakis, V. and Hughes, C. (1991) Nature 351, 759–761.

8 Pellet, S., Boehm, D.F., Snyder, I.S., Rowe, G. and Welch, R.A. (1990) Infect. Immun. 58, 822–827.
9 Ludwig, A., Vogel, M. and Goebel, W. (1987) Mol. Gen. Genet. 206, 238–245.
10 González-Carreró, M.I., Zabala, J.C., De la Cruz, F. and Ortiz, J.M. (1985) Mol. Gen. Genet. 199, 106–110.
11 Ostolaza, H., Bartolomé, B., Serra, J.L., De la Cruz, F. and Goñi, F.M. (1991) FEBS LETT. 280, 195–198.
12 Snyder, I.S. and Koch, N.A. (1966) J. Bacteriol. 91, 763–767.
13 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta 858, 161–168.
14 Ellens, H., Bentz, J. and Szoka, F.C. (1985) Biochemistry 24, 3099–3106.
15 Nieva, J.L., Goñi, F.M. and Alonso, A. (1989) Biochemistry 28, 7364–7367.
16 Hoekstra, D., de Boer, T., Klappe, K. and Wilschut, J. (1984) Biochemistry 23, 5675–5681.
17 Parente, R.A., Nir, S. and Szoka, F.C.Jr. (1990) Biochemistry 29, 8720–8728.
18 Weinstein, J.N., Klausner, R.D., Innerarity, T., Ralston, E. and Blumenthal, R. (1981) Biochim. Biophys. Acta 647, 270–274.
19 Benz, R., Schmid, A., Wagner, W. and Goebel, W. (1989) Infect. Immun. 57, 887–895.
20 Nagawa, Y. and Regen, S.L. (1992) J. Am. Chem. Soc. 114, 1668–1672.
21 Ojcius, D.M. and Young, J.D.E. (1991) Trends Biochem. Sci. 16, 225–229.
22 Levin, I.W. (1984) in Handbook of Natural Toxins, Vol. 2 (Tu, A.T., ed.), pp. 87–108, Marcel Dekker, Inc., New York.
23 Dawson, C.R., Drake, A.F., Helliwell, J. and Hidder, R.C. (1978) Biochim. Biophys. Acta 510, 75–86.
24 De Grado, W.F., Musso, G.F., Lieber, M., Kaiser, F. and Kezdy, F.J. (1982) Biophys. J. 37, 329–338.
25 Terwilliger, T.C., Weissmann, L. and Eisenberg, D. (1982) Biophys. J. 37, 353–361.
26 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207–218.
27 Tilcock, C.P.S., Bally, M.B., Farren, S.D. and Cullis, P.R. (1982) Biochemistry 21, 4596–4601.
28 Tilcock, C.P.S., Bally, M.B., Farren, S.D., Cullis, P.R. and Gruner, S.M. (1984) Biochemistry 23, 2696–2703.
29 Seddon, J.M. (1990) Biochim. Biophys. Acta 1031, 1–69.
30 Madden, T.D. and Cullis, P.R. (1982) Biochim. Biophys. Acta 684, 149–153.
31 Ludwig, A., Schmid, A., Benz, R. and Goebel, W. (1991) Mol. Gen. Genet. 226, 198–208.
32 Alonso, A., Villena, R. and Goñi, F.M. (1991) FEBS Lett. 123, 200–204.
33 García, L.A.M., Araujo, P.S. and Chaimovich, H. (1984) Biochim. Biophys. Acta 772, 213–216.