Biochimica et Biophysica Acta, 557 (1979) 156—169

BBA 78530

CLASSIFICATION OF MICROBIAL, PLANT AND ANIMAL CYTOLYSINS BASED ON THEIR MEMBRANE-DAMAGING EFFECTS ON HUMAN FIBROBLASTS

MONICA THELESTAM [a,*] and ROLAND MÖLLBY [b]

[a] *Department of Bacteriology, Karolinska Institute, S-104 01 Stockholm, and* [b] *National Bacteriological Laboratory, S-105 21 Stockholm (Sweden)*

(Received February 16th, 1979)

Key words; Cytolysin; Hemolysin; Membrane damage; Cytoplasmic leakage; Cytotoxicity; (Human fibroblast)

Summary

38 cytolytic agents of mainly microbial origin were investigated with respect to membrane-damaging activity on human diploid fibroblasts. Increased plasma membrane permeability was measured as leakage of three defined cytoplasmic markers of various sizes: α-aminoisobutyric acid, uridine nucleotides and ribosomal RNA. The relative leakages of these markers, caused by different concentrations of the various cytolysins, yielded a leakage pattern for each substance. Five distinct types of leakage patterns were obtained. These were transformed into numerical expressions by calculating the ratios between the amounts of cytolysin needed to release 50% of the nucleotide and ribosomal RNA markers and the amounts required to release 50% of the α-aminoisobutyric acid marker (ED_{50} ratios).

A classification of the cytolysins into five groups was arrived at on the basis of the different types of leakage patterns with the aid of reference cytolysins with well-known mechanisms of membrane interaction. These groups comprised: (1) detergent-like agents, (2) agents interacting with only certain constituents of the cell membrane, (3) agents interacting with specific receptor molecules in the membrane, (4) agents inducing small functional holes of a definable size, and (5) agents inducing only a very limited increase in plasma membrane permeability. The system may be useful for characterization and differentiation of new cytolytic agents of various sources as it divides membrane-damaging agents into separate groups on the basis of their principal function on intact human cells.

* To whom correspondence should be directed.

Introduction

Cytolysins can be defined as agents capable of causing physical dissolution of mammalian or other cells in vitro. Cytolytic agents are elaborated by a variety of organisms in nature, microorganisms as well as plants and animals. The basis for their cytolytic effects is their membrane-damaging activity which depends on a direct interaction between cytolysin and the plasma membrane of sensitive cells [1]. This interaction alters the chemical and/or structural configuration of the cell membrane, leading to changes in membrane permeability and finally lysis of the cell.

Some cytolysins enzymically hydrolyze the phospholipids of the cell membrane (phospholipases A and C) [2] while the majority are non-enzymic. For some of the latter cytolysins a receptor molecule in the membrane has been defined, although complete information regarding the precise mechanism of the membrane damage may still be lacking. In many other cases virtually nothing is known about the nature of the membrane interaction.

A seemingly relevant basis for classification of cytolysins is the traditional grouping according to origin, i.e. microbial, plant or animal. However, some cytolysins of widely different origins have a common membrane receptor. This may be exemplified with thiol-activated haemolysins (from different species of true bacteria), polyene antibiotics (from Streptomycetes), metridiolysin (from sea anemone) and some of the plant-derived saponins, which all bind to membrane sterols [3,4]. Sharing the same receptor, however, does not necessarily imply a common cytolytic process.

The chemical character of a cytolysin would constitute another ground for its classification. The above mentioned cholesterol-interactive agents represent rather diverse chemical structures; the thiol-activated hemolysins and metridiolysin are proteins [4], the polyene antibiotics are macrolides [5] and the saponins are glycosides of varying chemical compositions [6]. However, the precise chemical structures of many cytolysins have not yet been elucidated.

Bacterial cytolysins, which dominate in the present study, have traditionally been classified according to their modes of membrane interaction into: (i) thiol-activated (oxygen labile), (ii) phospholipases, (iii) surface active, and (iv) unknown mode of action [1,7].

The ability to produce lesions in the target-cell membrane is the most critical character of a cytolysin. It has been suggested that different kinetics of haemolysis displayed by different cytolysins are determined by the different types of primary lesions or holes * produced in the membrane [8,9]. The aim of the present work was thus to measure changes in the permeability of a human diploid cell membrane caused by various biological cytolysins, and to explore the possibility of using such membrane-damaging effects as a criterion for classification of cytolysins. To determine the type of primary membrane lesion, leakage of three differently sized radioactive markers from the cytoplasm of human diploid fibroblasts was measured [10—12]. The molecular size

* The modern concept of biomembranes as dynamic fluid structures seems to exclude the possibility of fixed holes present in the membrane. The word 'hole' is used herein as a purely operational term to indicate a change in membrane permeability allowing leakage of cytoplasmic substances.

of leaked marker(s) was considered indicative of the size of the functional holes induced in the membrane.

The various types of leakage patterns obtained with different cytolytic substances are described herein. A new classification scheme is suggested on basis of these patterns. In order to transform the patterns into numerical expressions, the relative sizes of the induced functional holes were estimated by the use of ED_{50} ratios.

Materials and Methods

Chemicals. Eagle's minimal essential medium and Hank's balanced salt solution were obtained from the National Bacteriological Laboratory, Stockholm, Sweden; trypsin was fromFlow Laboratories, Ltd., Irvine, Scotland, [5-^{3}H]-uridine (spec. act. >25 Ci/mmol), α-[1-^{14}C]aminoisobutyric acid (spec. act. >50 Ci/mol) and Aquasol ® Universal Cocktail from NEN Chemicals GmbH, Frankfurt, F.R.G.

TABLE I

CYTOLYTIC AGENTS OF MICROBIAL ORIGIN

If no source is given in the table, the agents were purified in our laboratory to at least the same degree of purity as obtained in the indicated reference of purification.

Origin	Toxin	Reference of purification	Source
Aeromonas hydrophila	Aerolysin	13	Å. Ljungh
	Cytotoxic protein	14	Å. Ljungh
Aspergillus fumigatus	Asp-haemolysin	15	K. Yokota
Bacillus cereus	Cereolysin	16	
	Phospholipase C	17	
Bacillus natto	Surfactin	18	Y. Kameda
Bacillus polymyxa	Polymyxin B	Not given	Sigma Chemicals
Clostridium perfringens	Phospholipase C (α-toxin)	19	
	θ-Toxin	20	
	δ-Toxin	21	J. Alouf
Corynebacterium ovis	Phospholipase D	R. Möllby and G. Blomquist/ unpublished	
Fucidium coccineum	Sodium fusidate	Not given	Leo Pharmaceuticals
	Derivative PR-1144 of sodium fusidate		Leo Pharmaceuticals
Listeria monocytogenes	Listeriolysin	22	
Pseudomonas aeruginosa	Haemolysin	23	Å. Ljungh
Staphylococcus aureus	α-Toxin	24	
	β-Toxin	25	
	γ-Toxin	25	
	δ-Toxin	26	
Streptococcus pyogenes	Streptolysin O	27	
	Streptolysin S	28	W. Hryniewicz
Streptomyces spp.	Filipin complex	Not given	Upjohn and Co.
	Nystatin	Not given	Squibb Institute
	Amphotericin B	Not given	Squibb Institute
	Methyl ester of amphotericin B	Semisynthetic	Squibb Institute
Vibrio parahaemolyticus	Vibriolysin	29	F. Takeda

TABLE II

CYTOLYTIC AGENTS OF PLANT AND ANIMAL ORIGIN

Origin	Toxin	Reference of purification	Source
Medicago sativa	Saponin (alfalfa)	30	S. Shany
Quillaja	Saponin (white)	Not given	BDH Chemicals Ltd.
Apis mellifera (bee venom)	Melittin	31	E. Habermann
Haemachatus haemachatus (cobra venom)	Direct lytic factor	32	D. Eaker
Naja naja (cobra venom)	Direct lytic factor	33	P.G. Lankish
	Phospholipase A	Crude venom	Sigma

Cytolytic agents. A total of 38 cytolysins were investigated. These are listed in Tables I—III with reference to source and purification procedures. Donators of purified substances and commercially not available pharmacological agents are gratefully acknowledged for their generous gifts.

Cultivation and labelling of cells. Human diploid embryonic lung fibroblasts (line MRC-5) were cultivated in Eagle's medium [34] in polystyrene wells to a cell density of 10^5 cells/cm^2. The cells were labelled with [^{3}H]uridine or α-[^{14}C]aminoisobutyric acid as previously described [10,11]. Labelling with aminoisobutyric acid was slightly modified as follows: monolayers were washed with Hank's balanced salt solution and then incubated with aminoisobutyric acid (1 μCi/ml) in the same medium at pH 7.4. After incubation for 60 min at 37°C this buffer was changed to Eagle's medium with serum, and the incubation at 37°C was continued for another 30 min. The cytoplasmic markers had the following molecular sizes: α-aminoisobutyric acid-label 103; nucleotide label <1000 and RNA-label >200 000.

Testing procedure and calculation of results. Labelled, washed confluent cell monolayers were incubated for 30 min at 37°C with cytolytic agents diluted in Tris-buffered saline using at least six different concentrations. Then the incubation media containing leaked cytoplasmic markers were removed and centrifuged (1000 × *g*, 10 min, 4°C), and radioactivity in 0.1-ml aliquots of the supernatants was measured by liquid scintillation as earlier described [10].

A maximal release of each marker was obtained by treating control cells for 30 min with a 0.06 M sodium borate buffer (pH 7.8) and scraping with a rubber policeman. This treatment ruptured the cell membrane leaving the

TABLE III

SYNTHETIC CYTOLYTIC AGENTS

Trade name	Chemical name	Source
Triton X-100	Polyoxyethyleneglycol(9-10)*p-t*-octylphenol	Rohm and Haas
Triton X-114	Polyoxyethyleneglycol(7-8)*p-t*-octylphenol	Rohm and Haas
—	Sodium dodecylsulphate (SDS)	E. Merck
	Sodium deoxycholate	E. Merck
Brij 35	Polyoxyethylenealcohol	E. Merck
Cetrimide	*N*-cetyl,*N*,*N*,*N*,-trimethylammonium bromide	E. Merck

nuclei intact [10]. The following formula was used to express the relative leakage of each marker:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

When the spontaneous release of any of the markers occasionally exceeded 30% the experiment was discarded. The spontaneous release during standard incubation (30 min at 37°C in Tris-buffered saline) was 3—7% of the maximal release in the case of RNA and nucleotide labels and 15—30% for the α-amino-isobutyric acid label.

Estimation of ED_{50} ratios. The effective dose releasing 50% of cytoplasmic label, ED_{50}, was calculated for each cytolytic agent by interpolation from the dose-response curves with the three different sized markers. From these ED_{50} values two kinds of ED_{50} ratios were calculated:

(a) nucleotide/α-aminoisobutyric acid, i.e. the ratio between the concentrations of cytolysin needed to release 50% of the nucleotide and α-aminoisobutyric acid labels, respectively, and

(b) RNA/α-aminoisobutyric acid, i.e. the ratio between the concentrations of cytolysin needed to release 50% of the RNA and α-aminoisobutyric acid labels, respectively.

Haemolytic and phospholipase C assays. Haemolytic activity of bacterial toxins was assayed as previously described [35,36]. Phospholipase activity was determined by a titrimetric method on egg yolk suspension as substrate [19].

Results

Different leakage patterns were produced with the various cytolysins and five groups were established on the basis of the character of these leakage patterns and the calculated ED_{50} ratios. Examples of leakage patterns typical of the five groups are presented in Fig. 1.

Group 1 (detergent group)

Substances comprising the first group (Fig. 1/1, Table IV) in duced functional holes large enough to permit an equal leakage of both the high molecular weight RNA label and the smaller nucleotide and α-aminoisobutyric acid labels. Thus, the three leakage curves ran closely to each other and ED_{50} ratios were calculated to about one.

All the examined detergents, except for cetyltrimethylammonium bromide, belonged to this group. Also the commercial *Quillaja* saponin and the cobra (*Naja naja*) venom extract, marketed by Sigma as phospholipase A, produced similar leakage patterns. Among substances of microbial origin the fungal antibiotic sodium fusidate was classified in Group 1, and the only bacterial cytolysin was the haemolysin from *Pseudomonas aeruginosa.* A typical representative of group 1 is seen in Fig. 1A. The leakage pattern of another common non-ionic detergent, Triton X-100, was presented earlier [12].

Group 2

Agents producing an almost equal leakage of the α-aminoisobutyric acid- and

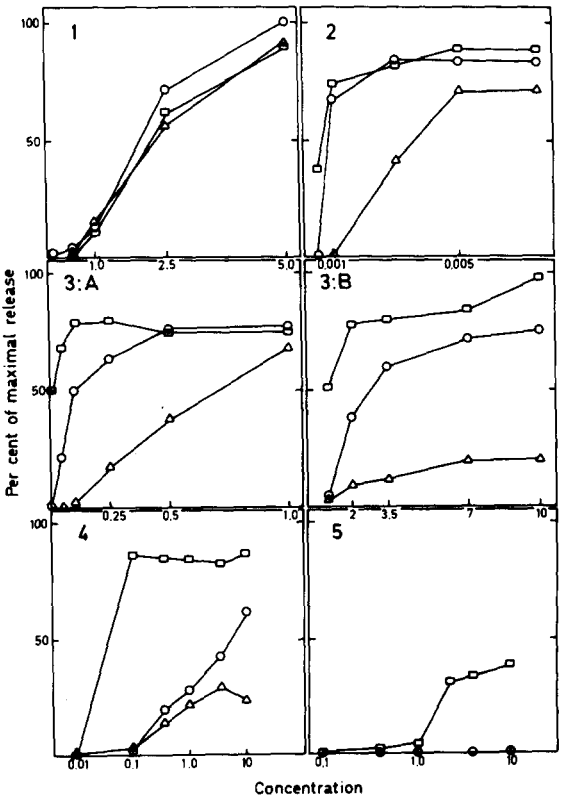

Fig. 1. Leakage patterns of Groups 1—5. RNA, nucleotide- and aminoisobutyric acid-labelled cells were treated for 30 min at 37°C with the test substances diluted in Tris-buffered saline (pH 7.0) at the concentrations indicated in the figure. The concentration are expressed as haemolytic units per ml in 1, 3 and 5, as % (w/v) in 2, and as μg/ml in 4. Note that the *x*-axes in 4 and 5 are logarithmic. Leakage of radioactivity was expressed as % of maximal release: 1. Haemolysin of *Ps. aeruginosa*; 2. alfalfa-saponin; 3A. δ-toxin of *S. aureus*; 3B. Asp-haemolysin; 4. nystatin; 5. δ-lysin of *C. perfringens*. □———□, α-aminoisobutyric acid label; ○———○, nucleotide label; △———△, RNA label.

TABLE IV

GROUP 1

Cytolytic agent	ED_{50} ratio nucleotide/ α-aminoisobutyric acid	ED_{50} ratio RNA/ α-aminoisobutyric acid
Pseudomonas haemolysin	0.9	1.0
Sodium-fusidate	1.4	1.9
Saponin (*Quillaja*)	0.8	1.1
Phospholipase A	0.6	0.9
Triton X-100	1.0	1.0
Triton X-114	1.0	1.0
SDS	1.2	0.9
Sodium deoxycholate	1.6	1.2
Brij 35	1.4	1.8
ED_{50} ratios range	0.6—1.6	0.9—1.9
median	1.0	1.0

TABLE V

GROUP 2

Cytolytic agent	ED_{50} ratio nucleotide/ α-aminoisobutyric acid	ED_{50} ratio RNA/ α-aminoisobutyric acid
Surfactin	4.8	6.1
Phospholipase C	2.1	4.4
Alfalfa-saponin	1.5	5.7
Melittin	3.6	8.2
ED_{50} ratios range	1.5—4.8	4.4—8.2
median	2.1	5.7

nucleotide labels, but a distinctly lower release of the RNA label at low concentrations, were classified in the second group (Fig. 1/2, Table V). In this group the RNA/α-aminoisobutyric acid ED_{50} ratios were somewhat higher than the nucleotide/α-aminoisobutyric acid ED_{50} ratios.

At higher concentrations of these cytolytic agents, the RNA label leaked out and the three curves approached each other. Cytolytic agents producing such leakage patterns may thus be said to cause somewhat smaller functional holes at low concentrations than the detergent-like agents (Group 1).

The basic polypeptide melittin from bee venom was assigned to this group, as well as the saponin purified from alfalfa roots. Two bacterial toxins were found in Group 2, viz. surfactin from *Bacillus natto* and phospholipase C from *Clostridium perfringens.* The leakage pattern caused by alfalfa-saponin (Fig. 1/2), illustrates the lowered leakage of RNA label as compared to that elicited by the detergent group.

Group 3

The agents classified in Group 3 (Fig. 1/3, Table VI) at low concentrations always caused a clearly lower release of nucleotide label than of α-aminoisobutyric acid label, and the RNA label was even more retained than with Group 2. This kind of leakage pattern results in nucleotide/α-aminoisobutyric acid ED_{50} ratios of around 5—15 and RNA/α-aminoisobutyric acid ED_{50} ratios in the region of 20 to more than 100. Thus the primary functional holes in the membrane, produced by this group of cytolytic agents were of considerably smaller dimensions than those induced by agents assigned to Groups 1 and 2. Many of the bacterial cytolysins belonged to this group.

The leakage patterns induced by the two first-mentioned agents in Table VI, aerolysin and staphylococcal δ-toxin, significantly differed from the majority of the agents in this group. The leakage curves of these cytolysins converged with increasing toxin concentrations (as exemplified with staphylococcal δ-toxin, Fig. 1/3A). By contrast, none of the other substances in Group 3 (as exemplified with the Asp haemolysin, Fig. 1/3B) caused a RNA leakage above 25% at the obtainable activities. In those cases the RNA/α-aminoisobutyric acid ED_{50} ratio was designated as exceeding the obtained quotient (Table IV). These differences in RNA leakage were not time-dependent since RNA label was released to the same extent even when the incubation time was prolonged up to 3 h (θ-toxin, Fig. 9 in Ref. 10).

TABLE VI

GROUP 3

Cytolytic agent	ED_{50} ratio nucleotide/ α-aminoisobutyric acid	ED_{50} ratio RNA/ α-aminoisobutyric acid
Aerolysin	15	26
δ-Toxin (*S. aureus*)	10	70
Cytotoxic protein	6	>40 *
Asp-haemolysin ***	3	>15
Cereolysin	12	40
θ-Toxin	8	>31
Listeriolysin	7	>70
Streptolysin O	7	>50
Filipin	1	>200
Virbriolysin	12	21 **
Direct lytic factor (*Haemachatus*)	4	>12
Direct lytic factor (*Naja*)	38	>100
Cetyltrimethylammonium bromide	3	>33
ED_{50} ratios ragne	1—38	26—>200
median	6	

* The figure for RNA/α-aminoisobutyric acid ED_{50} ratio was designated as exceeding the obtained quotient when it was not possible to release as much as 50% of the RNA label.

** ED_{10} ratios, i.e. ratios between the concentrations causing 10% release of the respective markers.

*** Incubated at 20°C which is the optimum temperature for the lytic activity of this haemolysin [15].

The classification of cereolysin and vibriolysin was not absolutely clear since the maximum available concentrations of these toxins were only 5 times as high as those releasing 50% of the α-aminoisobutyric acid label. Because of this ED_{10} ratios were given. This complication was due to the fact that, by contrast to the synthetic agents, the maximal available concentrations of biological cytolysins are limited by the specific activities obtained in each preparation.

Group 4

Group 4 (Fig. 1/4, Table VII) comprises cytolytic agents which caused release of α-aminoisobutyric acid label at low concentrations, whereas much higher concentrations were needed to release the nucleotide label. The RNA label was poorly released, if at all, even at high concentrations. Thus the ED_{50} ratios were very high and sometimes indeterminable since no leakage at all was detected

TABLE VII

GROUP 4

Cytolytic agent	ED_{50} ratio nucleotide/ α-aminoisobutyric acid	ED_{50} ratio RNA/ α-aminoisobutyric acid
α-Toxin (*S. aureus*)	>4	∞ *
Nystatin	132	>200
Amphotericin B	2500	5000
Amphotericin B methyl ester	>17	∞
ED_{50} ratio range	>4—2500	>200

* No RNA label was released.

TABLE VIII

GROUP 5

All ED_{50} ratios were ∞, i.e. only α-aminobutyric acid was released.

Phospholipase C (*B. cereus*)
Polymyxin B
δ-Toxin (*C. perfringens*)
Phospholipase D
Sodium fusidate derivative PR-1144
Phospholipase C (*S. aureus*; β-toxin)
γ-Toxin
Streptolysin S

(denoted as ∞). This leakage pattern reflects small functional holes in the cell membrane.

Two clinically uses polyene antibiotics belonged to this group together with *Staphylococcus* α-toxin. The leakage pattern of nystatin (Fig. 1/4) was very similar to that found for amphotericin B [12]. The leakage pattern of α-toxin was presented earlier [37]. The methylester hydrochloride derivative of amphotericin B was also assigned to this group although it differed significantly from the parent substance since no RNA release could be produced at any concentration tested [38].

Group 5

Substances releasing the α-aminoisobutyric acid label only were classified in Group 5 (Fig. 1/5, Table VIII), constituting the smaller extreme with regard to the size of induced functional holes. In this case the term hole is probably seriously misleading as the changes in the cell membrane permeability are very subtle. Indeed, these agents induce only few other detectable cytotoxic effects, which indicates the sensitivity of this membrane-specific test [12,39].

All the agents belonging to this group were of bacterial origin except for the semisynthetic derivative PR-1144 of sodium fusidate. The *S. aureus* γ-toxin was most active, releasing about 80% of the α-aminoisobutyric acid label at a concentration of 16 haemolytic units [37]. However, the other substances in this group gave rise to release of significant but limited amounts of the α-aminoisobutyric acid label as exemplified in Fig. 1/5 with the δ-lysin from *C. perfringens*.

Discussion

In the study of interactions between cytolysins and cell membranes, erythrocytes have hitherto been the preferred target-cell type. However, erythrocytes as a test system present unwanted variations which are avoidable in other cell systems. Erythrocytes necessarily come from different sources in different laboratories and furthermore the membrane composition of erythrocytes varies in relation to cell age [40]. In this investigation human diploid embryonic fibroblasts were used in order to present a 'normal' cell membrane of a metabolizing human cell to the cytolysins in a well-standardized test system [12].

The standard incubation time (30 min) was chosen so as to obtain optimal differences in leakage between the three cytoplasmic markers. Since interest

was focused on the primary lesions caused by the cytolysins it was important to keep the incubation time as short as possible, a factor crucial for the character of the leakage patterns obtained. 38 cytolysins were tested and classified into five groups according to leakage patterns illustrated in Fig. 1 and ED_{50} ratios presented in Tables IV—VIII.

Five of the six detergents tested were classified in Group 1, i.e. they induced large functional holes in the fibroblast plasma membrane. It should be noted that the induced holes were of large size over the entire range of membrane-damaging concentrations, i.e. also at concentrations causing no observable alterations of cellular morphology. This result is consistent with the fact that detergents extract membrane lipids and proteins, thereby disrupting the structural integrity of the membrane [41]. Although much work has been done to elucidate the mechanism of membrane interactions of selected detergents [41—43], there is no evident explanation of the fact that the cationic detergent cetyltrimethylammonium bromide did not induce any significant release of RNA label, suggesting that the functional holes were of small dimensions. An as yet unexplored possibility is that RNA label could not leak out because cetyltrimethylammonium bromide caused an accumulation of positive charges that inhibited the passage of RNA molecules.

The classification of commercial *Quillaja* saponin in Group 1 is in agreement with the well-known detergent activity of the non-cardiac active saponins [6]. The haemolysin from *Pseudomonas aeruginosa* was the only bacterial toxin associated with Group 1. This result is consistent with the surfactant activity of this glycolipid, which is capable of solubilizing various phospholipids [44,45].

The detergent-like effect of the phospholipase A from cobra venom was expected, since such crude preparations also contain direct lytic factor. This basic polypeptide and the phospholipase A exert a synergistic effect on cell membranes [46—48]. However, two purified preparations of direct lytic factor alone induced only a limited membrane lesion. This result is in accordance with Lankisch et al. [49], who demonstrated both an osmotic and non-osmotic component of direct lytic factor-induced haemolysis. Phospholipase A from different sources may vary considerably in its effects on cell membranes [50]. In our experiments a highly purified phospholipase A from *Naja nigricollis* (kindly supplied by D. Eaker, Uppsala) did not affect the membrane permeability of human fibroblasts (unpublished data).

The molecular configuration of sodium fusidate resembles that of the cholic acids. As might be expected from its structure the substance is strongly surface active [51] and in agreement with this it belonged to the 'detergent group'. However, the derivative PR-1144 of sodium fusidate was classified into Group 5, indicating that the detergent activity of the parent molecule had been completely removed.

Each substance in Group 1 has earlier been stated to have 'surfactant' or 'detergent-like' activity. Surface active polypeptides, however, showed different leakage patterns. The polypeptide melittin from bee venom has been shown to be surface active [52,53] by interacting with the fatty acids of membrane phospholipids [54]. However, its membrane interaction differed from that displayed by the agents in Group 1 since melittin induced a relatively lower release of RNA label. This result is in accordance with a recently proposed

model for the interaction of melittin with membranes, postulating that melittin causes a wedge effect in membranes, which can not be equated with the effects induced by simple detergents [55]. A similar leakage pattern was obtained with surfactin, a result in agreement with its amphipathic nature reminiscent of that of melittin [9,18].

The *C. perfringens* phospholipase C induced relatively large holes as compared to the other phospholipases C in this investigation. Indeed, the clostridial phospholipase C has the broadest substrate specificity of these enzymes, hydrolysing all ordinary phospholipids of the mammalian cell membrane. Phospholipase C from *B. cereus* hydrolyses the glycerophospholipids but not sphingomyelin. Staphylococcal β-toxin on the other hand, is specific for sphingomyelin [2]. The latter two phospholipases caused only subtle increases in the permeability of the fibroblast membrane (Group 5) which indicates that hydrolysis of both sphingomyelin and phosphatidylcholine is required to induce large functional holes. This is further supported by the fact that a combination of β-toxin and *B. cereus* phospholipase C, induced a similar type of lesion to that caused by clostridial phospholipase C [50,56].

Highly purified alfalfa-saponin is known to bind to membrane cholesterol. However, interaction of saponin with cholesterol is not dependent on a 3-β-hydroxyl group as is the case for both polyenes [57] and the thiol-activated haemolysins [7]. Assa et al. [58] suggested that breakdown of the erythrocyte membrane with alfalfa-saponins was caused not only through the binding to cholesterol but also because of non-specific interactions of saponins with membrane proteins and phospholipids. This may explain why the lesions induced with alfalfa-saponin (Group 2) in the fibroblast membrane were larger than those induced by the thiol-activated haemolysins (Group 3).

True surface active substances, with the exception of cetyltrimethylammonium bromide, were classified in Group 1, and the basic amphipathic polypeptides in Group 2. The surface active protein δ-toxin, however, at low concentrations caused even smaller holes. These differences might be correlated to the molecular sizes of the membrane-damaging agents. Furthermore, in the case of cetyltrimethylammonium bromide and δ-toxin, the smaller holes may be explained by the cationic nature of these cytolysins [25].

All the tested thiol-activated haemolysins (θ-toxin, streptolysin, listeriolysin and cereolysin), as well as the polyene antibiotic filipin-induced leakage patterns characterized by a low RNA release. These agents are thought to exert their cytolytic effect by interaction with membrane cholesterol [59—61]. However, the facts that the thiol-activated haemolysins share a common receptor and induce similar leakage patterns do not prove that the subsequent lytic events are identical (unpublished data).

Some recently purified cytolysins, the Asp haemolysin, cytotoxic protein from *Aeromonas hydrophila* and vibriolysin, induced leakage patterns of a similar character as the thiol-activated haemolysins. It may be speculated that the membrane interaction of these agents also involves a specific receptor molecule, limited in amount or availability in the membrane. However, the nature of possible receptors cannot be deduced from our results. Besides cholesterol, possible receptors are specific gangliosides, (glyco)proteins, or phospholipids. For example, the vibriolysin has been claimed to attach to gangliosides [62].

Nystatin and amphotericin B produced remarkably smaller functional holes in the fibroblast membrane than the related polyene filipin and were classified into Group 4. This result is in agreement with the current concept of polyene antibiotic membrane interaction, i.e. that filipin forms disruptive aggregates with cholesterol in the membrane interior, whereas nystatin and amphotericin B induce narrow transverse pores by stereochemically defined reactions with the membrane sterols [63—66]. Very high concentrations of nystatin and amphotericin B caused a significant release also of the RNA label. At these concentrations coalescence of the large number of transverse pores may destroy the permeability barrier function of the membrane. Alternatively, at high concentrations these polyenes might cause a more general membrane disorganization.

Staphylococcal α-toxin is surface active which may explain some of its membrane-damaging effects in different systems [67,68]. Its strongly haemolytic activity on rabbit erythrocytes is thought to be mediated by interaction with a high affinity glycoprotein receptor [69]. It is not known if such high affinity receptors are present on the human fibroblast surface. However, the increase in fibroblast membrane permeability induced by α-toxin was more probably due to its general surface activity since the same degree of membrane damage was induced in several other cell lines (unpublished data).

Two of the agents assigned to Group 5 were phospholipases C with rather narrow substrate specificities (as discussed above) and the phospholipase D, a specific sphingomyelinase [70]. Polymyxin B has been claimed to exert is membrane-damaging effect by specific reaction with certain phospholipids [71,72], while the mechanisms of membrane interaction displayed by streptolysin S, γ-toxin and the δ-lysin of *C. perfringens* have not been identified. Although the latter toxins are haemolytic they do not cause cytolysis when the membrane of a metabolizing cell is exposed to them, probably since the subtle membrane damage can be counteracted by an active repair [73].

This investigation has led to a classification of cytolytic agents based upon the characteristics of the membrane-damage they induce. This classification system may be of value for characterization and differentiation of new membrane-damaging agents. With a slight simplification, the groups could be characterized as follows.

1. Detergent-group comprising detergent-like agents which non-specifically solubilize the membrane constituents.

2. Substances interacting with only certain constituents of the cell membrane, either by solubilization or degradation.

3. Cytolysins interacting with specific receptor molecules in the cell membrane, thus giving rise to membrane lesions of limited size.

4. Substances which induce small holes of a definable size (e.g. pores with approx. 5 Å diameter).

5. Substances inducing only a very limited increase in the permeability of the plasma membrane.

However, this classification cannot be used for a direct characterization of membrane receptors or for elucidation of the final steps in the cytolytic process, although indirect evidence is obtained as discussed above. The proposed system may be used to assign a wide variety of biological cytolysins

and other membrane-damaging agents into separate categories on the basis of their principal function.

Acknowledgements

We thank Cyril Smyth for linguistic advice, and critical comments on this manuscript. Expert technical assistance by Greger Blomquist, Eva Brännkärr, Christina Hedström, Lena Norenius and Gunnel Sigstam is gratefully acknowledged. J. Alouf, D. Eaker, E. Habermann, W. Hryniewicz, Y. Kameda, P.G. Lankisch, Å. Ljung, S.J. Lucania, S. Shany, F. Takeda, L. Tybring, G.B. Whitfield and K. Yokota are gratefully acknowledged for their generous supply of test substances. This investigation was supported by grants from the Swedish Medical Research Council (16X-2562), Nordiska Samfundets Stiftelse and Karolinska Institutets funds.

References

1 Bernheimer, A.W. (1974) Biochim. Biophys. Acta 344, 27—50
2 Möllby, R. (1978) in Bacterial Toxins and Cell Membranes (Jeljaszewicz, J. and Wadström, T., eds.), pp. 367—424, Academic Press, London
3 Shany, S., Bernheimer, A.W., Grushoff, P.S. and Kim, K.S. (1974) Mol. Cell. Biochem. 3, 179—186
4 Bernheimer, A.W., Avigad, L.S. and Kim, K.S. (1979) Toxicon 17, 69—75
5 Kinsky, S.C. (1970) Annu. Rev. Pharmacol. 10, 119—142
6 Birk, Y. (1969) in Toxic Constituents of Plant Food Stuffs (Liener, I.E., ed.), pp. 169—210, Academic Press, New York
7 Alouf, J.E. (1976) in The Specificity and Action of Animal, Bacterial and Plant Toxins (Cuatrecasas, P., ed.), pp. 219—270, John Wiley, New York
8 Bernheimer, A.W. (1947) J. Gen. Physiol. 30, 337—353
9 Bernheimer, A.W. (1970) in Microbial Toxins (Ajl, S.J., Kadis, S. and Montie, T.C., eds.), Vol. 1, pp. 183—212, Academic Press, New York
10 Thelestam, M. and Möllby, R. (1975) Infect. Immun. 11, 640—648
11 Thelestam, M. and Möllby, R. (1975) Infect. Immun. 12, 225—232
12 Thelestam, M. and Möllby, R. (1976) Med. Biol. 54, 39—49
13 Wretlind, B., Möllby, R. and Wadström, T. (1971) Infect. Immun. 4, 503—505
14 Ljungh, Å., Wretlind, B. and Wadström, T. (1978) in Toxins, Animal, Plant and Microbial (Rosenberg, P., ed.), pp. 947—960, Pergamon Press, Oxford
15 Yokota, K., Shimada, H., Kamaguchi, A. and Sakaguchi, O. (1977) Microbiol. Immunol. 21, 11—22
16 Bernheimer, A.W. and Grushoff, P. (1967) J. Gen. Microbiol. 46, 143—150
17 Little, C., Aurebekk, B. and Otnaess, A.-B. (1975) FEBS Lett. 52, 175—179
18 Kameda, Y., Ouhira, S., Matsui, K., Kanatomo, S., Hase, T. and Atsusaka, T. (1974) Chem. Pharm. Bull. 22, 938—944
19 Möllby, R. and Wadström, T. (1973) Biochim. Biophys. Acta 321, 569—584
20 Smyth, C.J. (1975) J. Gen. Microbiol. 87, 219—238
21 Tixier, G. and Alouf, J.E. (1976) Ann. Microbiol. (Inst. Pasteur) 127B, 509—524
22 Jenkins, E.M., Njoku-Obi, A.N. and Adams, E.W. (1964) J. Bacteriol. 88, 418—424
23 Berk, R.S. (1964) J. Bacteriol. 88, 559—565
24 Wadström, T. (1968) Biochim. Biophys. Acta 168, 228—242
25 Möllby, R. and Wadström, T. (1973) in Topics in Microbiology and Immunology (Jiljaszewicz, J., ed.), Vol. 11, pp. 298—313, Karger, Basel
26 Kreger, A.S., Kim, K.S., Zaboretzky, F. and Bernheimer, A.W. (1971) Infect. Immun. 3, 449—465
27 Smyth, C.J. and Fehrenbach, F.J. (1974) Acta Pathol. Microbiol. Scand. Sect. B 82, 860—870
28 Hryniewicz, W., Gray, E.D., Tagg, J.R., Wannamaker, L.W., Kanclerski, K. and Laible, N. (1978) Zbl. Bakt. Hyg. I. Abt. Orig. A, in the press
29 Honda, T., Taga, S., Takeda, T., Hasibuan, M.A., Takeda, Y. and Miwatani, T. (1976) Infect. Immun. 13, 133—139
30 Shany, S., Birk, Y., Gestetner, B. and Bondi, A. (1970) J. Sci. Food Agric. 21, 131—135
31 Habermann, E. and Reiz, K.G. (1965) Biochem. Z. 341, 451—466
32 Fryklund, L. and Eaker, D. (1973) Biochemistry 12, 661—667

33 Lankisch, P.G. and Vogt, W. (1972) Biochim. Biophys. Acta 270, 241—247
34 Eagle, H. (1959) Science 130, 432—437
35 Thelestam, M., Möllby, R. and Wadström, T. (1973) Infect. Immun. 8, 938—946
36 Strandberg, K., Möllby, R. and Wadström, T. (1974) Toxicon 12, 199—208
37 Thelestam, M. (1976) Zentralbl. Bakteriol. Parasitenkd. Infectionskr. Hyg. Abt. 1, Suppl. 5, 679—690
38 Thelestam, M. and Möllby, R. (1978) Current Chemother. 498—500
39 Szmigielski, S., Janiak, M., Möllby, R., Wadström, T. and Jeljaszewicz, J. (1978) Toxicon 16, 567—574
40 Van Gastel, C., van den Berg, D., de Gier, J. and van Deenen, L.L.M. (1965) Br. J. Haemat. 11, 193—199
41 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29—79
42 Philippot, J. (1971) Biochim. Biophys. Acta 225, 201—213
43 Simons, K., Helenius, A. and Garoff, H. (1973) J. Mol. Biol. 80, 119—133
44 Sierra, G. (1960) Antonie van Leeuwenhoek 26, 189—192
45 Kurioka, S. and Liu, P.V. (1967) J. Bacteriol. 93, 670—674
46 Condrea, E., de Vries, A. and Mager, J. (1964) Biochim. Biophys. Acta 84, 60—73
47 Klibansky, C., London, Y., Frenkel, A. and de Vries, A. (1968) Biochim. Biophys. Acta 150, 15—23
48 Vogt, W., Patzer, P., Lege, L., Oldigs, H.-D. and Wille, G. (1970) Naunyn-Schmiedeberg's Arch. Pharmakol. 265, 442—454
49 Lankisch, P.G., Damerau, B. and Vogt, W. (1974) Naunyn-Schmiedeberg's Arch. Pharmakol. 282, 255—260
50 Colley, C.M., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 307, 74—82
51 Stewart, G.T. (1964) Pharmakotherapia 2, 137—148
52 Habermann, E. and Jentsch, J. (1967) Hoppe-Seylers Z. Physiol. Chem. 348, 37—50
53 Sessa, G., Freer, J.H., Colacicco, G. and Weissman, G. (1969) J. Biol. Chem. 244, 3575—3582
54 Williams, J.C. and Bell, R.M. (1972) Biochim. Biophys. Acta 288, 255—262
55 Dawson, C.R., Drake, A.F., Helliwell, J. and Hider, R.C. (1978) Biochim. Biophys. Acta 510, 75—86
56 Thelestam, M. and Möllby, R. (1978) XIIth Int. Congr. Microbiol., p. 196 (Abstract) (Int. Assoc. Microbiol. Soc.)
57 Norman, A.W., Demel, R.A., de Kruijff, B., Geurts van Knessel, W.S.M. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 290, 1—14
58 Assa, Y., Shany, S., Gestetner, B., Tencer, Y., Birk, Y. and Bondi, A. (1973) Biochim. Biophys. Acta 307, 83—91
59 Bernheimer, A.W. (1976) in Mechanisms in Bacterial Toxinology (Bernheimer, A.W., ed.), pp. 85—97, John Wiley, New York
60 Smyth, C.J. and Duncan, J.L. (1978) in Bacterial Toxins and Cell Membranes (Jeljaszewicz, J. and Wadström, T., eds.), pp. 129—182, Academic Press, London
61 Tillack, T.W. and Kinsky, S.C. (1973) Biochim. Biophys. Acta 323, 43—54
62 Takeda, Y., Takeda, T., Honda, T., Sakurai, J., Ohtomo, N. and Miwatani, T. (1975) Infect. Immun. 12, 931—933
63 Verkleij, A.J., de Kruijff, B., Gerritsen, W.F., Demel, R.A. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 291, 577—581
64 Andreoli, T.E. (1974) Ann. N.Y. Acad. Sci. 235, 448—468
65 De Kruijff, B. and Demel, R.A. (1974) Biochim. Biophys. Acta 339, 57—70
66 Van Hoogevest, P. and de Kruijff, B. (1978) Biochim. Biophys. Acta 511, 397—407
67 Buckelew, A.R. Jr. and Colacicco, G. (1971) Biochim. Biophys. Acta 233, 7—16
68 Freer, J.H. and Arbuthnott, J.P. (1976) in Mechanisms in Bacterial Toxinology (Bernheimer, A.W., ed.), pp. 169—193, John Wiley, New York
69 Cassidy, P. and Harshamn, S. (1976) Biochemistry 15, 2348—2355
70 Soucek, A., Michalec, C. and Souckova, A. (1971) Biochim. Biophys. Acta 227, 116—128
71 Feingold, D.S., Hsu Chen, C.C. and Sud, I.J. (1974) Ann. N.Y. Acad. Sci. 235, 480—492
72 Hartmann, W., Galla, H.-J. and Sackmann, E. (1978) Biochim. Biophys. Acta 510, 124—139
73 Warren, L. and Glick, M.C. (1968) J. Cell Biol. 37, 729—746