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Interactions of antibiotics of the iturin group with human erythrocytes

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The peptidolipid antibiotics, iturin A and bacillomycin L have a disrupting effect on erythrocyte membrane leading to a simultaneous release of K^+ ions and hemoglobin. The formation of ghosts is accompanied by a partial solubilisation of lipid components. Binding experiments with radioactive antibiotics show that about $7 \cdot 10^7$ molecules of iturin A and $4 \cdot 10^7$ molecules of bacillomycin L are bound to one erythrocyte at the concentration giving 100% hemolysis. This concentration is reduced by about 20% after treatment of erythrocytes by phospholipase A_2. Scatchard plots show that the affinity for erythrocyte membrane is higher with bacillomycin L than with iturin A. This difference is probably correlated to the differences in their peptide moieties. The substitution of tyrosyl residue leads to a ten-fold increase of the concentrations giving 100% hemolysis, probably due to a low distribution coefficient of derivatives between the membrane and the solvent. This result and the humped Scatchard curves obtained with both antibiotics may be related to the self-association of the compounds in aqueous solutions.

Introduction

Iturin A and bacillomycin L are peptidolipid antibiotics produced by various strains of *Bacillus subtilis* [1]. These compounds are characterized by the presence of a liposoluble β-amino acid [2] linked to a peptide moiety containing L- and D-α-aminoacids [3,4] giving a cyclic structure. According to the nature of α-amino acids, there are two types of antibiotics of iturin group: anionic antibiotics such as bacillomycin L which contains a carboxylic group belonging to an aspartyl residue [5] and neutral antibiotics such as iturin A which contains no carboxylic group but amide groups of asparagine residues.

These antibiotics are potent antifungal agents, they interact with fungal cytoplasmic membrane thus creating transmembrane channels which permit the release of vital ions [6–8]. Unfortunately, they also interact with red cells causing hemolysis [9] and this is probably the basis of their toxicity.

To understand the mode of action of these antibiotics it is important to obtain information on their binding to the membranes, probably to the lipid phase of the membranes. In the present work we studied first the permeability changes of the human erythrocyte membrane induced by two antibiotics of iturin group in comparison with those induced by amphotericin B, a polyene antibiotic and then the relation between the structures of antibiotics, their binding to the erythrocyte membrane and their hemolytic activities.

Material and Methods

Antibiotics and chemicals

Iturin A and bacillomycin L were obtained and purified as previously described [10]. Amphotericin B, phospholipase A_2 (bee venom), bovine serum albumin (fatty acid-free) were purchased

from Sigma (U.S.A.), Soluene and Dimilume 30 from Packard Instrument (France).

K^+ ions and hemoglobin release from erythrocytes

Fresh human blood was collected on 3.8% sodium citrate, centrifuged at $3000 \times g$ for 10 min and the plasma and buffy coat were discarded. The erythrocytes were washed three times with 0.15 M NaCl and suspended in an equal volume of saline. An aliquot (0.1 ml) of the erythrocyte suspension was incubated with 9.9 ml of a 0.15 M NaCl solution containing increasing amounts of antibiotics (final concentration: $2.5 \cdot 10^7$ cells/ml as determined in Malassez hematimeter). After incubation at 37°C for 30 min the suspensions were centrifuged, an aliquot of the supernatant was measured for its K^+ ions content with a flame photometer (Elvi 660) and the hemoglobin release was determined by measuring the absorbance at 540 nm.

Lipids analysis of the supernatants

1 ml of the packed red blood cells, in 50 ml of 0.15 M NaCl solution, was incubated for 30 min at 37°C with 1.5 mg of bacillomycin L or 2.5 mg of iturin A. After centrifugation, 2 h at $20000 \times g$, the lipids of the supernatant (25 ml) were extracted by 100 ml of chloroform/methanol (2 : 1, v/v). Another aliquot (25 ml) was filtered on a Millipore filter (0.45 μm) before extraction with solvents.

The analysis of lipids was made in comparison with lipids extracted from packed erythrocytes according to Gibson et al. [11]. The lipid fractions were analyzed by thin-layer chromatography on silica gel 60 plates. The following developing solvents were used: hexane/ether/acetic acid (70 : 30 : 1, v/v) for cholesterol and chloroform/methanol/propan-1-ol/0.25% KCl/ethyl acetate (25 : 13 : 25 : 9 : 15, v/v) [12] for phospholipids. Quantitative analysis of cholesterol was carried out by gas chromatography on a glass column 3% OV 17 on chromosorb WAW DMCS 80/100 at 280°C, 0.15 MPa (Intersmat). For quantitative analysis of phospholipids the chromatograms were sprayed with a solution of 3% cupric acetate in 8% phosphoric acid [13]. After charring by heating in an oven at 180°C for 10 min, the chromatograms were scanned on a Joyce-Loebl densitometer (chromoscan 200) using the reflexion mode.

Preparation of tritium-labeled antibiotics

Preparation of iodo-derivatives of antibiotics. 20 μmol of ICl dissolved in methanol were slowly added to 10 μmol of antibiotic in 10 ml of 0.1 M phosphate buffer, at pH 7.2 for iturin A or at pH 8.8 for bacillomycin L, at 20°C; after 1 min an excess of sodium thiosulfate was added to destroy the unreacted iodine.

The mixture was lyophilized and passed on a Sephadex LH 20 column (18 cm × 1.7 cm) to remove the salts and traces of iodine. Elution was made with hexane/chloroform/methanol (25 : 45 : 10, v/v). Then the iodo-derivatives were separated from non-halogenated antibiotics by preparative thin-layer chromatography on silica gel 60 in chloroform/methanol/water (65 : 25 : 4, v/v), R_F iodoiturin A = 0.45, R_F iodobacillomycin L = 0.21. The products were recovered from the plate by methanol. Ultraviolet spectra showed two maxima at $\lambda = 285$ nm and 295 nm corresponding to monoiodinated and diiodinated derivatives, respectively.

Tritiation. Iodoiturin A (0.95 μmol) and iodobacillomycin L (0.97 μmol) were each dissolved in 1 ml of pure methanol and solutions were then frozen. The catalyst (palladium oxide: 10–11 mg) was dispersed on the surface and the reacting flasks connected to the automatic gas handling device [14]. While keeping the flask frozen, a vacuum of 10^2 Pa was established, then the pure tritium gas (60 Ci) made by the Commissariat à l'Energie Atomique was introduced and compressed to 10^5 Pa. The catalyst was maintained 15 min in contact with the gas on top of the solution still frozen. After thawing, the reaction mixture was kept at room temperature under constant magnetic stirring for 2 h. The absorbance of tritium gas produced a reduction in pressure of about $8 \cdot 10^4$ Pa and $3 \cdot 10^4$ Pa for iturin A and bacillomycin L, respectively. Palladium oxide was removed from the reacting mixture by filtration over Millipore (GS) filter and labile tritium atoms eliminated by successive flash evaporation with a large volume of dilute methanol (methanol/water, 50 : 50, v/v). Total radioactivities recovered were: 4.36 mCi (iturin A), 9.27 mCi (bacillomycin L).

The ^{3}H-labelled antibiotics (crude materials) were analyzed using thin-layer chromatography on silica gel with chloroform/methanol/water (65: : 25 : 4, v/v). The autoradiochromatograms and ^{3}H-scannings performed on each compound revealed a predominant peak ($R_F = 0.29$ for iturin A, $R_F = 0.08$ for bacillomycin L) comigrating with the references and corresponding to the spot detectable with the reagent spray: 4,4′-tetramethyldiaminodiphenylmethane. Then, the preparative chromatograms were separated into successive strips and the antibiotics extracted from silicagel powder sonicated with 5 × 5 ml of pure methanol. After centrifugation and concentration, the ultraviolet light spectra of tritiated antibiotics were found to be exactly the same as that of the original material ($\lambda_{max} = 275$ nm) for both compounds. Specific radioactivities were found to be: iturin A, 20 Ci/mmol; bacillomycin L, 40 Ci/mmol.

For binding experiments the pure labeled antibiotics were diluted with unlabeled antibiotics. To cover the concentration range in binding experiments two preparations of ^{3}H-labeled antibiotics with different activities were used: 0.4 Ci/mmol for the smallest concentrations and 0.04 Ci/mmol for the largest concentrations. In the following, ^{3}H-labeled antibiotics design a mixture of unlabeled and radioactive antibiotic at the suitable activity.

Preparation of radioactive O-methyltyrosine derivatives of antibiotics

A mixture of ^{3}H-labeled and unlabeled antibiotics was submitted to methylation by diazomethane as previously described [15]. This methylation gave the *O*-methyltyrosineiturin A but with bacillomycin L which possesses one carboxylic group and one phenolic group this treatment gave the *O*-methyltyrosinebacillomycin L methyl ester. The *O*-methyltyrosinebacillomycin L was obtained by mild saponification with 0.025 M NaOH for 1 h at 50°C. These derivatives were purified as previously described [15].

Binding experiments

Increasing amounts of ^{3}H-labeled antibiotics in 900 µl of 0.15 M NaCl were incubated with 100 µl of the erythrocyte suspension (final concentration: $5 \cdot 10^7$ cells/ml). After a 30 min incubation at 37°C, aliquots (50 µl) of the suspension were counted. Then, the erythrocyte suspension was centrifuged at 12 000 × *g* for 15 min and the radioactivity of supernatants aliquots (50 µl) was measured. All assays were made in polypropylene tubes and controls showed no retention of radioactive antibiotic on the tube wall. The amount of antibiotic bound to the membranes was calculated as the difference between the total radioactivity and the radioactivity in the supernatant.

Similar experiments were performed with various amounts of erythrocytes for antibiotic concentrations giving 100% hemolysis.

Measure of radioactivity: the samples were incubated in Soluene (400 µl) during 2 h. Then isopropanol (100 µl) and H_2O_2 (200 µl) were added and the mixture was kept for 30 min at room temperature. After mixing with 4 ml of Dimilume 30 the radioactivity was counted in a Packard spectrometer. The efficiency of the counting was about 30%.

Gel permeation chromatography

To 500 µl of the erythrocyte suspension were added 200 µg of [^{3}H]iturin A or 150 µg of [^{3}H]bacillomycin L in solution in 0.15 M NaCl, final volume 1 ml and final concentration $2.5 \cdot 10^8$ erythrocytes/ml. After a 30 min incubation at 37°C and centrifugation, the ghosts resulting from the action of the antibiotics were suspended in 500 µl of 0.15 M NaCl solution, put on a column of Sepharose 2B (19 cm × 2 cm) previously saturated with ghosts membranes and eluted with the same solution. The detection was performed in a LKB spectrophotometer (2138 Uvicord) coupled with a recorder. An aliquot of each fraction was taken for the radioactivity measurement.

Action of antibiotics on ghosts

The ghosts were prepared according to Burton et al. [16] and suspended in 0.15 M NaCl, the suspension contained about $3 \cdot 10^8$ ghosts/ml. Aliquots of the ghosts suspension were incubated for 30 min at 37°C with iturin A or [^{3}H]iturin A in 0.15 M NaCl, final volume 1 ml ($2 \cdot 10^7$ ghosts/ml). Then the ghosts suspensions were centrifuged for 15 min at 40 000 × *g*. The pellets were counted or resuspended in saline for washings.

Treatment of erythrocytes by phospholipase A_2

Erythrocytes were treated by phospholipase A_2 as described by Haest et al. [17]. After incubation at 37°C for 1 h and removal of the enzyme by centrifugation, the erythrocytes were incubated with antibiotics as described above.

Determination of antagonisation by bovine serum albumin

Each tube contained the antibiotic at the concentration giving 100% hemolysis (25 μg/ml for iturin A and 15 μg/ml for bacillomycin L) and bovine serum albumin in a molar ratio 1:1. To this mixture were added erythrocytes in saline (final volume 1 ml and $5 \cdot 10^7$ cells/ml). Hemolysis was measured after incubation of erythrocytes 30 min at 37°C and centrifugation.

Results

Permeability changes induced in erythrocytes

The permeability changes induced by antibiotics of iturin group in erythrocytes were compared with those induced by a polyene antibiotic amphotericin B, for low and high molecular weight compounds, K^+ ion and hemoglobin. In the presence of 4 μg/ml of amphotericin B, 50% of intracellular K^+ were found outside the erythrocytes while no hemoglobin was released (Fig. 1A). Thus the release of K^+ was not directly related to hemolysis. This result was similar to those previously reported [18,19] and it agreed with the mechanism of action which had been proposed for amphotericin B [20].

In the case of antibiotics of iturin group, after incubation with various amounts of iturin A or bacillomycin L a simultaneous release of hemoglobin and K^+ was observed (Figs. 1B, 1C). Thus the permeability changes induced by antibiotics of iturin group were not selective and the disruption of the erythrocytes membrane permitted the release of all intracellular compounds whatever their molecular weight.

Effect of phospholipase A_2 treatment on the hemolytic activity

Fresh erythrocytes were treated by phospholipase A_2 as described by Heast et al. [17]. Erythrocytes were then incubated with iturin A or bacillomycin L and hemolysis was measured. The results are presented in Fig. 2. The antibiotic concentrations required for hemolysis were reduced of about 20 to 30%.

Removal of lipid

In all experiments the lipids released from erythrocytes after action of antibiotics were compared with those obtained in the absence of antibiotic (NaCl control). The control supernatants contained 0.22% of total cholesterol and 0.1% of total phospholipids. At a concentration giving 100% hemolysis iturin A and bacillomycin L induced the release of 2.8% and 1.9% of cholesterol and 4.2% and 2.3% of phospholipids, respectively.

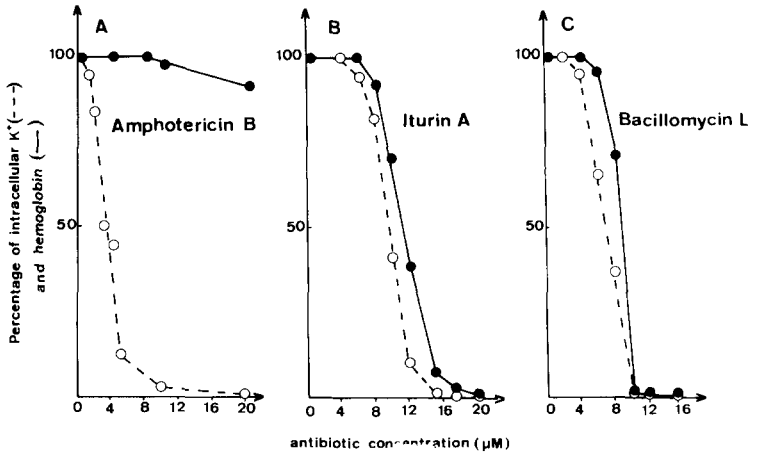

Fig. 1. Intracellular K^+ and hemoglobin after a 30 min incubation at 37°C of $2.5 \cdot 10^7$ erythrocytes/ml with increasing amounts of antibiotics. The results are expressed as percentages of total intracellular K^+ and hemoglobin.

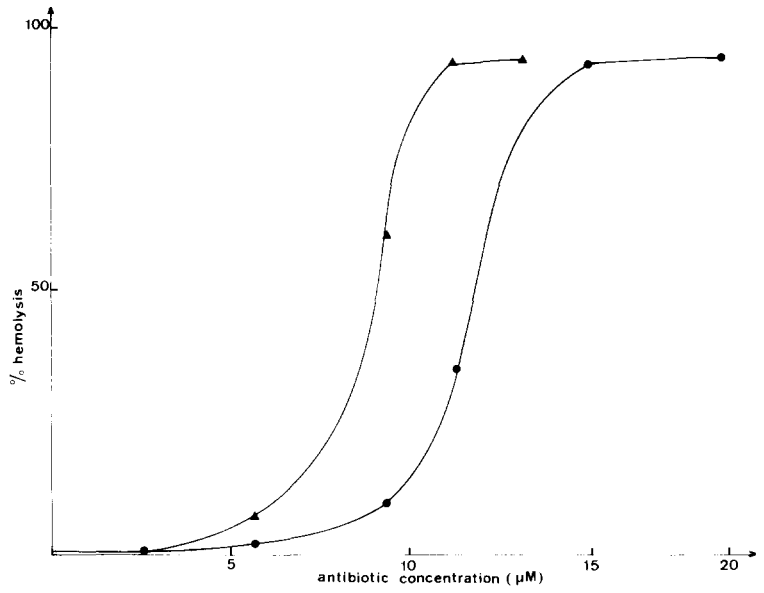

Fig. 2. Effect of phospholipase A_2 treatment of erythrocytes on hemolytic activity of bacillomycin L. $5 \cdot 10^7$ intact erythrocytes (●) or treated erythrocytes (▲) are incubated with increasing amounts of bacillomycin L. After a 30 min incubation at 37°C hemoglobin is measured in the supernatant. The results are expressed as percentages of total lysis.

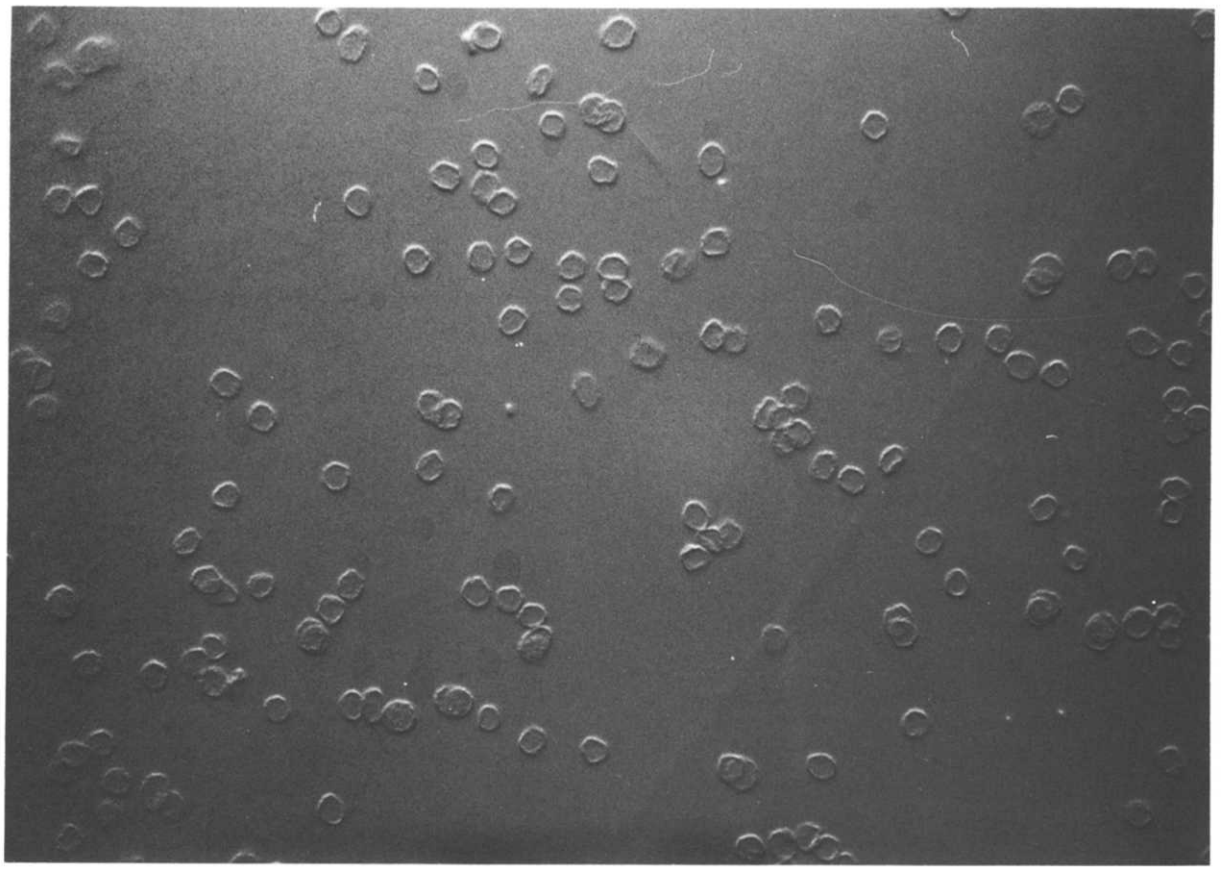

Fig. 3. Differential-interference contrast microscopy of ghosts obtained after incubation of erythrocytes with a concentration of iturin A giving 100% hemolysis (×830).

The released phospholipids had a distribution which was not significantly different from those of the phospholipids of intact erythrocytes.

Thus the interaction of both antibiotics with erythrocytes gives a disturbance of the cytoplasmic membrane with a partial release of the lipid components but this action does not seem to have a specificity towards a special type of lipids.

Association of antibiotics with the ghosts resulting from hemolysis

Hemolytic concentrations of iturin A or of bacillomycin L were added to suspensions of erythrocytes. After hemolysis and release of the intracellular components the assays were centrifuged; the white pellets were washed once with cold isotonic saline and suspended in the same solvent. The examination of the suspensions by phase contrast microscopy showed the formation of ghosts, homogeneous in size as shown in Fig. 3.

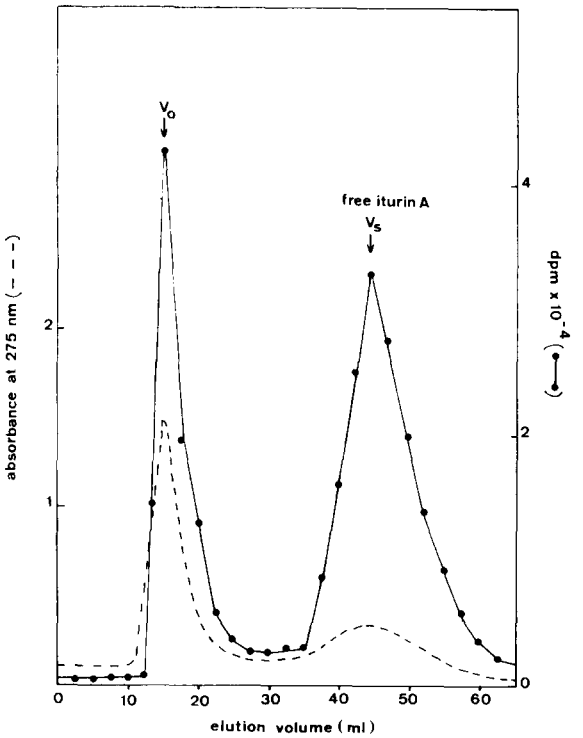

Fig. 4. Elution profile on Sepharose 2B of ghosts obtained with a hemolytic concentration of [^{3}H]iturin A in 0.15 M NaCl solution. ------, absorbance at 275 nm (arbitrary units); ●——●, radioactivity in dpm. V_0, void volume; V_s, solvent volume.

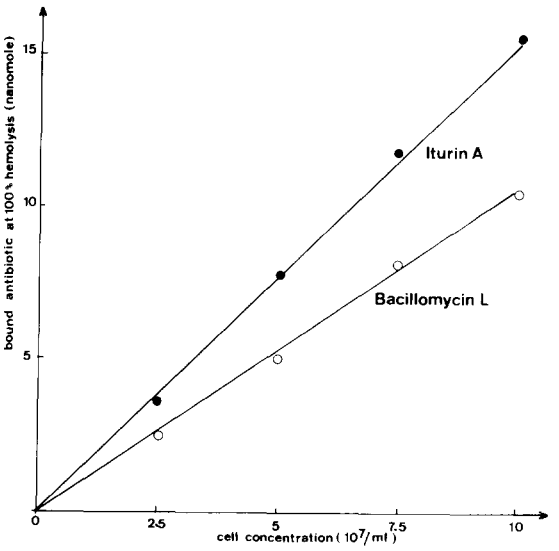

Fig. 5. Membrane-bound antibiotic at 100% hemolysis in relation to cell concentration. Assays contain various erythrocytes concentrations and the corresponding amount of ^{3}H-labeled antibiotic giving 100% hemolysis. After a 30 min incubation at 37°C the radioactivity in the pellet is measured as described in Materials and Methods. Bound antibiotic is expressed in nmol per total pellet.

In order to demonstrate the retention of antibiotics on ghosts the hemolysis was performed with [^{3}H]iturin A or [^{3}H]bacillomycin L. The suspension of ghosts was passed through a column of Sepharose 2B, the eluates were studied by spectrophotometry at 275 nm for the presence of ghosts and of the antibiotic and by measurement of the radioactivity for the location of the antibiotic. The elution profile obtained with [^{3}H]iturin A is given in Fig. 4. The first fraction, $V_e = 15$ ml contained ghosts and bound antibiotic while the unbound antibiotic was eluted in the last fraction, $V_e = 45$ ml.

Influence of the cell concentration on the binding

The influence of the antibiotic/cell ratio on the antibiotic binding was studied by measuring the bound antibiotic for various amounts of erythrocytes at antibiotic concentrations giving 100% hemolysis. The results are shown in Fig. 5. The amount of bound antibiotic is a linear function of the erythrocyte concentration, therefore the bound antibiotic/cell ratio is constant for all the erythrocyte concentrations tested. Thus complete

TABLE I

BINDING OF ^{3}H-LABELED ANTIBIOTICS TO ERYTHROCYTES AFTER A 30-min INCUBATION AT 37°C

The concentration giving 100% hemolysis was 25 μg/ml for iturin A and 15 μg/ml for bacillomycin L with $5 \cdot 10^7$ erythrocytes/ml. Bound antibiotic was expressed in μg per total cell pellet.

Amount of antibiotic (μg/ml)	Percentage in the pellet [a]		Bound antibiotic	
	iturin A	bacillomycin L	iturin A	bacillomycin L
1	9	11	0.09	0.11
2	10	21	0.20	0.42
3	17	25	0.5	0.7
5	22	28	1.1	1.4
6	23	32	1.4	1.9
8	25	34	2.0	2.7
10	26	35	2.6	3.5
12	28	35	3.1	4.2
15	28	35	4.2	5.2
20	30	34	6.0	6.8
25	31	32	7.7	8.0
30	31	29	9.3	8.7
40	28	26	11.2	10.4
50	27	24	12.5	12.0
80	23	17	18.4	13.6
100	22	14	22.2	14.0
150	17	10	26.0	15.0
200	16	8	32.0	16.0

[a] Values are the mean of four or five independent assays.

hemolysis does occur only when a definite number of antibiotic molecules is bound to a cell, this number varies with the nature of the antibiotic: at the 100% hemolysis concentration, about $7 \cdot 10^7$ molecules of iturin A and $4 \cdot 10^7$ molecules of bacillomycin L were retained per erythrocyte.

Influence of the antibiotic concentration on erythrocyte binding

The binding to erythrocytes was studied in a large range of antibiotic concentrations, below and above the 100% hemolysis concentration. The results are summarized in Table I. The amount of bound antibiotic increased with the total antibiotic added to the erythrocyte suspension and the highest percentage of bound antibiotic was found for the concentration giving 100% hemolysis. This percentage was quite similar for iturin A and bacillomycin L, 31% and 35%, respectively.

When the data were analyzed by Scatchard plots (Fig. 6) the curves showed positive slopes with maxima corresponding to the 100% hemolysis concentrations and then, the curves had negative slopes. From these data the maximal amounts of bound antibiotics may be determined: $5.7 \cdot 10^8$ molecules and $2.2 \cdot 10^8$ molecules per ghost for iturin A and bacillomycin L, respectively.

Desorption of the antibiotic from the ghosts

Several ghosts suspensions were incubated with

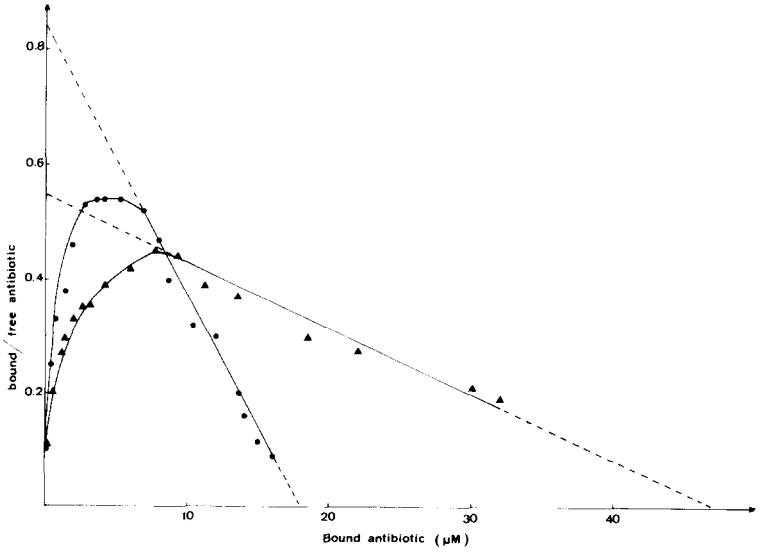

Fig. 6. Scatchard plots of binding of [^{3}H]iturin A (▲——▲) and [^{3}H]bacillomycin L (●——●) after a 30 min incubation at 37°C.

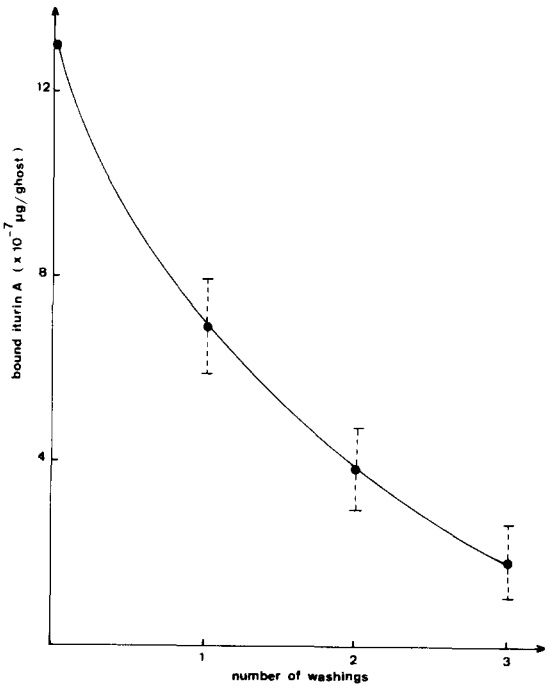

Fig. 7. Binding of [^{3}H]iturin A to ghosts after successive washings. About $2 \cdot 10^7$ ghosts/ml are incubated with 20 µg of [^{3}H]iturin A for 30 min at 37°C. After centrifugation the pellets are counted or resuspended in 0.15 M NaCl for washings.

[^{3}H]iturin A and the pellets obtained after centrifugation were submitted to several washings. The results are shown in Fig. 7. In each washing 40 to 50% of the bound antibiotic was solubilized, thus the binding of the antibiotic to ghosts was very labile and an equilibrium was established between bound antibiotic on ghosts and free antibiotic in NaCl solution.

This equilibrium was confirmed by incubating a ghosts suspension either with unlabeled iturin A or with radioactive iturin A (60 µg). After centrifugation the pellets were resuspended in 1 ml of 0.15 M NaCl containing 20 µg of either radioactive or unlabeled iturin A, respectively. After standing for 15 min, the suspensions were centrifuged at 40000 $\times g$, then the pellets and the supernatants were counted. In the first assay 33% of bound iturin A was exchanged with radioactive iturin A and in the other assay 50% of the radioactive iturin A was released from the ghosts.

Influence of the tyrosine residue of antibiotics on their hemolytic and binding properties

The presence of a tyrosine residue was found essential for the antifungal activity of iturin A and bacillomycin L [21] and this activity was strongly diminished when the tyrosine residue was *O*-methylated. *O*-Methyltyrosineiturin A and *O*-methyltyrosinebacillomycin L were tested for their hemolytic activities and their binding to erythrocytes. The concentrations giving 100% hemolysis were about 10-fold higher than that of natural antibiotics: 250 µg and 140 µg for *O*-methyl derivatives of iturin A and of bacillomycin L, respectively, instead of 25 µg and 15 µg for non-derivatized antibiotics. The amount of bound compounds at hemolytic concentrations was about 3-folds higher with *O*-methyl derivatives than with natural antibiotics and the balance between bound and total antibiotic was quite different for substituted iturin A : 8% and bacillomycin L : 12% in comparison with unsubstituted iturin A : 31% and bacillomycin L : 35%.

The great difference observed between the concentrations of *O*-methyl derivatives and those of antibiotics required for hemolysis may be due to a very low distribution coefficient of derivatives between membrane and solvent.

Antagonisation of hemolytic activity by bovine serum albumin

When hemolysis was measured in the presence of bovine serum albumin the activity of iturin A was strongly reduced (70%) while hemolysis was only reduced of 20%, in the presence of bacillomycin L.

Discussion

Antibiotics of iturin group are potent hemolytic agents. Our purpose was to study the action of two antibiotics of this group, a neutral compound, iturin A and an anionic one, bacillomycin L. Both antibiotics are amphipathic molecules and their action is localized on the membrane of erythrocytes. Two processes could occur in the membrane: either the formation of channels of increasing sizes with increasing concentrations of antibiotics or a rapid drastic disruption of the membrane giving rise to large pores allowing the release of

hemoglobin. With iturin A and bacillomycin L the studies of the released K^+ ions and hemoglobin showed that both materials were lost at the same rate. In comparison low concentrations of amphotericin B permitted the release of K^+ ions only, as a consequence of the formation of small channels permeable only to low-molecular weight compounds. The disruption of the erythrocyte membrane by the antibiotics of iturin group is therefore drastic, it might be due to a detergent effect in the lipid domain going along with the creation of structural defects which would cause the release of the macromolecular compounds and the solubilisation of lipids.

The action of the antibiotics was investigated more precisely by studing their binding to erythrocytes and to ghosts issued from erythrocytes. In a suspension containing $5 \cdot 10^7$ erythrocytes the 100% hemolysis concentrations were 25 μg/ml (25 μM) for iturin A and 15 μg/ml (15 μM) for bacillomycin L corresponding, respectively, to $9 \cdot 10^7$ and $6 \cdot 10^7$ molecules of iturin A and bacillomycin L per erythrocyte. After 100% hemolysis a further binding of antibiotics to ghosts was observed and Scatchard curves gave the theoretical maximum amounts of antibiotics which were bound per ghost: $5.7 \cdot 10^8$ molecules of iturin A and $2.2 \cdot 10^8$ molecules of bacillomycin L. These values are closely related to the total number of lipid molecules per cell (approx. $5 \cdot 10^8$/cell), it seems probable that some sort of superficial loose attachment, possibly even of micelles is responsible for much of binding. Scatchard plots go through a maximum, they are humped or convex upward. These shapes are characteristic of ligand-ligand interaction [22]: the ligands attract each other and the binding is positively cooperative. This cooperativity is higher for bacillomycin L than for iturin A. Such self-association has already been found in a related group of pepetidolipids by NMR studies [23] and preliminary results showed a high autoaffinity for iturin A (unpublished results). Scatchard curves also showed a higher affinity of bacillomycin L for erythrocytes in agreement with the fact that this antibiotic has a minimal hemolytic concentration lower than that of iturin A. The differences of binding between iturin A and bacillomycin L may be related to their structures. Both antibiotics have similar lipid moieties but they differ in their peptide sequence. As a consequence their conformations, as shown by NMR analysis, are different (Ref. 24, and unpublished results) and the spatial forms of the molecules are obviously an essential parameter in their interaction with the membrane.

When erythrocytes were treated with phospholipase A_2 the hemolytic concentrations of both antibiotics were lowered to 70% of their normal values. Phospholipase A_2 does not destroy the cell membrane and the products of its action, lysophosphatidylcholines and fatty acids, stayed put and maintained a permeability barrier to hemoglobin [25]. However, the disturbance in the lipid composition modifies the accessibility of the membrane to various drugs and it was found that melittin solubilized an important amount of phospholipids only after treatment of erythrocytes by phospholipase A_2 [26]. The diminution of the hemolytic concentration for iturin A and bacillomycin L after action of phospholipase could be due to a looseness of the membrane allowing a better interaction with antibiotics.

When bovine serum albumin was added to antibiotics the hemolytic activity was strongly reduced in the case of iturin A, 30% of the normal value, but this protective effect was less effective in the case of bacillomycin L: hemolysis was still 82% of the normal value. On the contrary, when the action of the antibiotics was studied on fungal cells, it was shown that bovine serum albumin totally inhibited the antifungal activity of bacillomycin L, while it only reduced that of iturin A by 20% (unpublished results). Presumably bovine serum albumin competes for the antibiotics but this competition depends on the binding affinity of the drug for mammalian or fungal cells. In fact, as stated above, the minimal hemolytic concentration for bacillomycin L is smaller (15 μg/ml) than that for iturin A (25 μg/ml) whereas the minimal inhibitory concentrations against *Saccharomyces cerevisiae* vary in inverse order: 60 μg/ml for bacillomycin L and 20 μg/ml for iturin A [6].

O-Methyltyrosine derivatives of iturin A and bacillomycin L were previously shown to be inactive against yeast cells [21]. The hemolytic activity is also strongly lowered (10-fold) when tyrosine residues are substituted. It is not easy to correlate such a slight modification of the structures with

such a strong disturbance in the activities against erythrocyte membranes and fungal cells. Because the primary structure of the peptide cycle cannot be to only factor to explain the difference in the affinities of those compounds, the substitution of the hydroxyl group of tyrosine may have an important consequence on the spatial conformation and auto-association of the antibiotics in accordance with preliminary results in NMR studies. The physical state, size and shape of their micelles, is certainly an essential factor in the interactions of the antibiotics. Physical studies are currently in progress to bring further evidence.

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