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ACTION OF PEPTIDOLIPIDIC ANTIBIOTICS OF THE ITURIN GROUP ON ERYTHROCYTES

EFFECT OF SOME LIPIDS ON HEMOLYSIS

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Iturin A, bacillomycin L and bacillomycin L dimethyl ester have a strong lytic activity upon human erythrocytes while iturin C is totally inactive. The hemolytic action of the antibiotics is inhibited by free cholesterol as well as by cholesterol included in mixed liposomes of phosphatidylcholine-cholesterol and to a lesser extent by phosphatidylcholine liposomes. This inhibition is the result of an interaction between the antibiotic and added lipids which diminishes the concentration of free antibiotic available to lyse erythrocytes. The inhibitory effect of liposomes on hemolysis demonstrates the affinity of the antibiotic for artificial membranes, especially those containing cholesterol.

Introduction

Antibiotics of the iturin group are peptidolipids of related structures (Fig. 1) [1–3]. Among the compounds of the group, iturin A, bacillomycin L and a derivative, bacillomycin L dimethyl ester have an antibacterial activity against a few strains of bacteria and a potent antifungal activity against a large variety of yeasts and fungi [4]. This antifungal activity is inhibited by various lipids: phospholipids, ergosterol and especially cholesterol [4]. Such inhibitions by sterols were previously observed with polyene antibiotics known to act upon the cytoplasmic membrane of microorganisms [5,6]. Similarly, the antibiotics of the iturin group were found to act upon cytoplasmic membranes [7,8]. Thus, protoplasts from *Micrococcus luteus* are lysed by iturin A and by bacillomycin L dimethyl ester while protoplasts from *Bacillus subtilis* have a more moderate sensitivity and spheroplasts from *Escherichia coli* are totally resistant towards all the antibiotics of the iturin group. These results suggest that the composition of the membrane is of great importance for the antibiotic sensitivity and the strong activity of antibiotics of the iturin group against yeasts and fungi could be related to the presence of sterols in their cytoplasmic membrane.

To elucidate the role of sterols in the membrane sensitivity, we studied the action of antibiotics of the iturin group upon the membrane of erythrocytes which contain high amounts of cholesterol. Four compounds were tested, iturin A, bacillomycin L, bacillomycin L dimethyl ester which have a high antifungal activity and iturin C which has no antifungal activity [3]. In a second step we studied the effect of cholesterol added to the system erythrocytes-antibiotic either in alcoholic solution or included in liposomes.

Material and Methods

Antibiotics and chemicals. Iturin A, iturin C, bacillomycin L and bacillomycin L dimethyl ester were obtained and purified as described previously

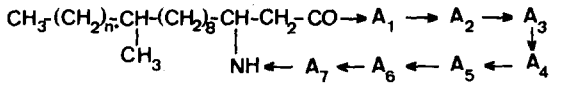

Fig. 1. Structures of antibiotics of the iturin group. *$n=0$, 3-amino-12-methyltridecanoic acid; *$n=1$, 3-amino-12-methyltetradenoic acid.

Antibiotic	A_1	A_2	A_3	A_4	A_5	A_6	A_7
Iturin A	L-Asn	D-Tyr	D-Asn	L-Gln	L-Pro	D-Asn	L-Ser
Iturin C	L-Asp	D-Tyr	D-Asn	L-Gln	L-Pro	D-Asn	L-Ser
Bacillomycin L	L-Asp	D-Tyr	D-Asp	L-Ser	L-Gln	D-Ser	L-Thr
Bacillomycin L dimethyl ester	L-Asp $\mid\beta$ $COOCH_3$	D-Tyr	D-Asp $\mid\beta$ $COOCH_3$	L-Ser	L-Gln	D-Ser	L-Thr

[3,9–11]. Phosphatidylcholine was purified from egg yolk according to Singleton et al. [12] and cholesterol was purchased from Prolabo (France).

Preparation of liposomes. Phosphatidylcholine (110 mg) or the mixture phosphatidylcholine-cholesterol (100 mg and 10 mg, respectively) in solution in chloroform were dried under a nitrogen atmosphere. Liposomes were prepared by sonicating the dried film in 10 ml of 0.05 M Tris-HCl buffer (pH 8.5) containing 0.10 M NaCl under a nitrogen atmosphere at 4°C on a 500 W MSE apparatus, sonicating tip diameter 2 cm. Liposomes thus obtained were used without further fractionation.

Determination of erythrocyte sensitivity to antibiotics. Human erythrocytes were prepared as described previously by Hsu-Chen and Feingold [13]. In each assay, antibiotics were added to 0.1 ml of erythrocyte suspension and the volume was adjusted to 1 ml with the NaCl solution; the final suspension contained $2.5 \cdot 10^6$ cells/ml. After a 30 min incubation at 37°C, the samples were centrifuged and the extent of hemolysis was determined by the measure of absorbance of the supernatant at 540 nm. The experimental data were corrected for the release of hemoglobin observed in the absence of antibiotics. The 100% hemolysis value was obtained by diluting 0.1 ml of the erythrocyte suspension with 0.9 ml of distilled water.

Determination of hemolysis in the presence of cholesterol. Assays were prepared by incubating 10 µl of a 1 mg/ml solution of bacillomycin L or 20 µl of a 1 mg/ml solution of iturin A with increasing volumes (from 1 to 16 µl) of a 5 mg/ml solution of cholesterol in ethanol; the volume was adjusted to 900 µl with a 0.15 M NaCl solution, then 100 µl of the erythrocyte suspension was added to the antibiotic-cholesterol mixture. After a 30 min incubation at 37°C, the samples were centrifuged and the absorbance of the supernatants was measured at 540 nm.

Determination of hemolysis in the presence of liposomes. The determination of the influence of liposomes on the hemolytic activity was performed as described above with cholesterol but a liposome suspension was used instead of the cholesterol solution.

The influence of the concentration of iturin A (20 µg/ml to 320 µg/ml) on the hemolytic activity was determined in a mixture of $2.5 \cdot 10^6$ erythrocytes and 160 µl of the phosphatidylcholine-cholesterol liposome suspension. The influence of the amount of erythrocytes was determined by adding $0.6 \cdot 10^6$ to $7.5 \cdot 10^6$ erythrocytes to 1 ml of 0.15 M NaCl containing either iturin A (20 µg/ml) or bacillomycin L (10 µg/ml) and an amount of phosphatidylcholine-cholesterol liposomes sufficient to inhibit completely hemolysis. Incubation conditions and measure of hemolysis were as described above.

Results

Action of antibiotics on erythrocytes

The sensitivity of fresh human erythrocytes in 0.15 M NaCl to various concentrations of iturin A, iturin C, bacillomycin L and bacillomycin L dimethyl ester was tested by measuring the hemoglobin released in the supernatant. The results are given in Fig. 2.

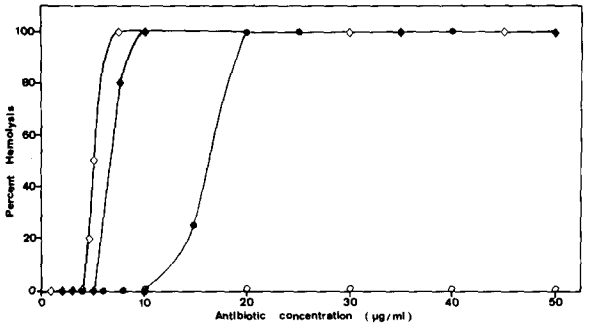

Fig. 2. Release of hemoglobin from human erythrocytes produced by antibiotics of the iturin group. The results are expressed as percentages of total lysis. Bacillomycin L (◆ ——— ◆), bacillomycin L dimethyl ester (◇ ——— ◇), iturin A (● ——— ●) and iturin C (○ ——— ○).

Iturin A, bacillomycin L and bacillomycin L dimethyl ester exhibit a strong hemolytic activity at 20 μg/ml, 10 μg/ml and 7.5 μg/ml, respectively, while iturin C, which is inactive upon microorganisms, has no more activity upon erythrocytes. Hemolysis was a very rapid event, it occurred in less than 1 min after addition of the antibiotic. In the limit of the experimental conditions, from 1 min to 2 h the percentage of hemoglobin released remained constant. Thus an incubation time of 30 min was arbitrarily chosen.

TABLE I

HEMOLYSIS OF ERYTHROCYTES BY ANTIBIOTICS IN THE PRESENCE OF CHOLESTEROL

$2.5 \cdot 10^6$ cells are incubated in 1 ml of isotonic solution with antibiotic (iturin A 20 μg/ml, bacillomycin L 10 μg/ml) and increasing amounts of cholesterol in solution in ethanol. After incubation, hemolysis is measured and the results are expressed as percentages of total hemolysis.

Antibiotic	Cholesterol (μg/ml)				
	5	10	20	40	80
Iturin A (20 μg/ml)	75	6	2	2	2
Bacillomycin L (10 μg/ml)	100	14	4	2	2

Effect of lipids on the hemolytic activity

The hemolytic activity of iturin A and bacillomycin L was tested in the presence of cholesterol. The cholesterol solution being at 5 mg/ml the percentage of ethanol in the assays never exceeded 1.6% of the final volume, it was verified that such a solution did not alter the erythrocytes. The antibiotics were used at the minimal concentration giving 100% of hemolysis. The results are reported in Table I.

TABLE II

HEMOLYSIS OF ERYTHROCYTES BY ANTIBIOTICS IN THE PRESENCE OF LIPOSOMES

Experiment is similar to that described in the legend of Table I except that the lipid antagonist was either a phosphatidylcholine liposome suspension (concentration: 11 μg/μl) or a phosphatidylcholine-cholesterol liposome suspension (concentration: 10 μg/μl phosphatidylcholine, 1 μg/μl cholesterol). Results are expressed as percentages of total hemolysis.

Antibiotic	μl of phosphatidylcholine liposome suspension						
	5	10	20	40	80	160	320
Iturin A (20 μg/ml)	100	94	83	41	28	3	3
Bacillomycin L (10 μg/ml)	100	93	83	9	2	2	2
	μl of phosphatidylcholine-cholesterol liposome suspension						
	5	10	20	40	80	160	320
Iturin A (20 μg/ml)	87	73	44	13	5	2	2
Bacillomycin L (10 μg/ml)	60	34	19	2	2	2	2

A strong inhibition was observed from a 10 μg/ml concentration of cholesterol. Another lipid, phosphatidylcholine, was tested for its inhibitory effect of the hemolytic activity of antibiotics. Phosphatidylcholine was used as liposomes and added to antibiotics and erythrocyte suspension. The action on hemolysis is given in Table II.

The inhibition of hemolysis by phosphatidylcholine occurred for much higher lipid concentrations than with cholesterol. When cholesterol was included in liposomes with phosphatidylcholine (ratio 1 : 10, w/w) the inhibition of hemolysis was higher than with phosphatidylcholine liposomes (Table II).

The inhibition of hemolytic effect by free cholesterol could probably be due to an interaction between antibiotic and cholesterol giving an inactive complex. In the presence of liposomes the mechanism is more complicated: either a liposome-antibiotic association could trap antibiotic molecules which would no more interact with erythrocytes or a liposome-erythrocyte association could prevent the attack of erythrocytes by antibiotics. The mechanism was studied by the measure of hemolysis with various amounts of antibiotics and of erythrocytes.

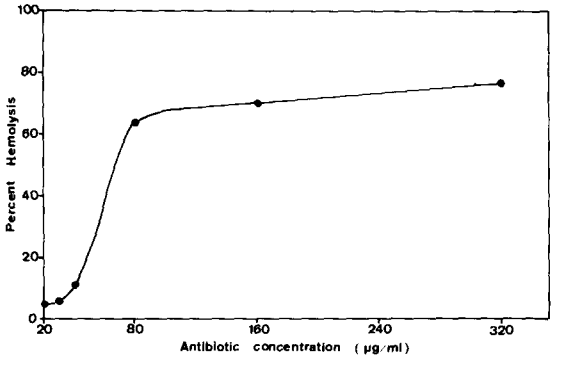

Fig. 3. Release of hemoglobin from human erythrocytes incubated with liposomes and increasing amounts of iturin A. Assays contain erythrocytes ($2.5 \cdot 10^6$ cells/ml), the amount of liposomes (160 μl) sufficient to inhibit hemolysis caused by 20 μg of iturin A and increasing concentrations of iturin A, from 20 to 320 μg/ml. After incubation the extent of hemolysis is measured. Results are expressed as percentages of total lysis.

TABLE III

HEMOLYSIS OF VARIOUS CONCENTRATIONS OF ERYTHROCYTES BY ANTIBIOTICS IN THE PRESENCE OF MIXED LIPOSOMES

Asays contain antibiotics and liposomes (20 μg/ml of iturin A and 160 μl of liposomes or 10 μg/ml of bacillomycin L and 80 μl of liposomes) in 1 ml of isotonic solution. Various amounts of erythrocytes are added and after incubation hemolysis is determined. The results are expressed as percentages of total hemolysis.

Antibiotic	Number of erythrocytes/ml ($\times 10^{-6}$)					
	0.6	1.2	1.7	2.5	5	7.5
Iturin A (20 μg/ml)	41	16	6	0	0	1
Bacillomycin L (10 μg/ml)	24	0	0	0	0	0

Influence of iturin A and erythrocyte concentrations on hemolysis

Erythrocytes ($2.5 \cdot 10^6$) were added to a mixture containing phosphatidylcholine-cholesterol liposomes and increasing amounts of iturin A. The hemolysis was measured and the results are given in Fig. 3.

The hypothesis of a protection of the erythrocyte surface by liposomes was ruled out by the reappearance of hemolysis when the antibiotic concentration increased. In fact, it seemed that liposomes would entrap antibiotic molecules until saturation, then an excess of antibiotic could interact with erythrocytes.

In other assays iturin A or bacillomycin L was added to phosphatidylcholine-cholesterol liposomes. The amount of liposomes just inhibited the hemolysis of erythrocytes in a suspension of $2.5 \cdot 10^6$ cells/ml. Various volumes of erythrocyte suspension were added and the hemolysis was measured. The results are given in Table III.

No or slight hemolysis occurred for concentrations of erythrocytes above $(1.2\text{–}1.7) \cdot 10^6$/ml but significant hemolysis was observed with lower concentrations of erythrocytes.

Discussion

The activity of the peptidolipids from the iturin group upon erythrocytes is quite parallel to their

antibiotic activity: iturin A, bacillomycin L, bacillomycin L dimethyl ester are hemolytic agents, they have also an antifungal activity and an antibacterial activity against *Micrococcus luteus* while iturin C has no hemolytic, antifungal or antibacterial activities. However iturin A and bacillomyin L dimethyl ester were found to have a lytic activity [14]. Thus the composition of the cytoplasmic membrane has an essential role in the interaction membrane-peptidolipids. The presence of a sterol seems to be a positive factor in this interaction as all peptidolipid antibiotics are potent antifungal agents: moreover the role of cholesterol seems very important in the hemolytic activity.

By adding cholesterol, either in free form, or included in liposomes a high inhibition of the hemolytic activity was observed. When liposomes contained only phosphatidylcholine without cholesterol a smaller inhibition occurred. Moreover this inhibition could be suppressed when higher amounts of antibiotics or smaller amounts of erythrocytes were used. These results are consistent with the following explanation. Peptidolipids of the iturin group have a high affinity for membranes containing sterols, especially cholesterol. In the presence of erythrocytes and vesicles, antibiotics had the highest affinity for artificial membrane, probably due to a less sophisticated arrangement than in natural membrane and the interaction antibiotic-vesicles superseded the interaction antibiotic-erythrocytes. With a higher antibiotic concentration, residual antibiotics could interact with erythrocytes and gave hemolysis. When the amount of erythrocytes decreased, the concentration of residual antibiotic became sufficient to disturb the erythrocyte membrane and to give hemolysis.

In conclusion, the mode of action of peptidolipid antibiotics from the iturin group is closely related to the lipid composition of cytoplasmic membrane of sensitive cells. The studies of hemolytic activity and of the inhibition of this activity show that sterols have an essential role in the antibiotic-membrane interaction.

Acknowledgements

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