Biological Activity of Amoebicin m4-A from
Bacillus licheniformis M-4

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Amoebicin m4-A from Bacillus licheniformis M-4 exerts a bactericidal and bacteriolytic action on Bacillus megaterium GR10. Protein, DNA, and RNA synthesis are inhibited, and the membrane electrical potential of this bacterium is depleted by amoebicin. Synthesis of DNA and RNA by Naegleria fowleri HB-1 is also inhibited. Liposomes constructed from l-α-phosphatidylcholine become permeable to ions, low-molecular-weight solutes, and high-molecular-weight polymers after treatment with amoebicin.

The amoeba Naegleria fowleri has been identified in the past as the causative agent of primary amoebic meningoencephalitis (1-3, 7, 21), a fulminating disease of the central nervous system occurring in adults with a recent history of swimming in warm waters and freshwaters (ponds, lakes, or rivers) which this amoeba inhabits (6, 11, 12, 18, 19, 20, 22).

Three strains of Bacillus licheniformis (A12, D-13, and M-4) from soil samples were selected in our laboratory for their marked amoebicidal and amoebolytic activity against human pathogenic and nonpathogenic strains of Naegleria (5). Strain A12 produced two amoebolytic peptide antibiotics (9). Moreover, three amoebolytic peptides (named amoebicins m4-A, m4-B, and m4-C) different from those produced by strain A12 were isolated from the spent medium of B. licheniformis M-4 and characterized (16).

The biological effects of peptide m4-A, the predominant inhibitor produced by strain M-4, have been studied in order to establish its mode of action. The results concerning its effects on Bacillus megaterium GR10, amoebae, and aseolin vesicles are presented in this paper.

MATERIALS AND METHODS

Microorganisms, growth conditions, and source of amoebicin. N. fowleri HB-1 (ATCC 30174) was maintained axenically in Cerva’s medium (3) at 37°C and subcultured every 72 h. Assays of biological activity were carried out with Cline medium (17). B. megaterium GR10 (from our laboratory collection) was grown in brain heart infusion (BHI) broth (BBL, Cockeysville, Md.) or Trypticase soy agar (BBL). B. licheniformis M-4, producer of amoebicin, was cultivated as described elsewhere (5). Purified amoebicin m4-A was obtained from culture supernatants of B. licheniformis M-4 by ammonium sulfate precipitation and reversed-phase chromatography as described previously (16).

Effect of amoebicin on B. megaterium GR10. Cultures were grown in BHI broth to an optical density at 620 nm of 0.15 absorbance units, and then different concentrations of purified amoebicin were added. The optical densities of controls and treated cultures were measured with a Spectronic 20 spectrophotometer, and the number of viable cells was determined by plating serial dilutions on Trypticase soy agar plates.

Incorporation of radioactively labeled precursors of macromolecular synthesis. To mid-log-phase cultures of B. megaterium GR10 (optical density at 620 nm = 0.1) were added the radioactive precursors [6-3H]thymidine, [5,6-3H]uridine, and [1-4,5-3H]leucine at a final concentration of 3 μCi/ml (1 Ci = 37 GBq). When the optical density at 620 nm reached 0.2 absorbance units, amoebicin m4-A in 10 mM Tris-HCl buffer (pH 7.0) (10 μg/ml, final concentration) or Tris-HCl buffer alone was added. Duplicate samples (200 μl each) were removed periodically from each tube and diluted in 4 ml of ice-cold 7% trichloroacetic acid (TCA). The precipitate was collected on glass fiber filters (GF/C; Whatman Ltd., Maidstone, England) and washed with 10 ml of ice-cold 7% TCA. The filters were dried and placed in vials to which scintillation fluid was added. Radioactivity was measured with a Beckman LS705 scintillation counter. All radioactive material was supplied by Amer sham International (Buckinghamshire, England).

Trophozoites of N. fowleri HB-1 grown in tissue culture flasks were resuspended in fresh Cline medium at 108 amoebae per ml in the presence of the radioactive precursors mentioned above (10 μCi/ml). After 2 h of incubation, the cultures were split into two aliquots to which amoebicin m4-A (10 μg/ml, final concentration) or Tris-HCl buffer was added. Duplicate samples (400 μl each) of control and treated cultures were placed on 24-well flat-bottom microtiter plates (16-mm diameter; Corning Glass Works, Corning, N.Y.). At regular intervals of incubation, 150 μl of 50% NaOH and 50 μl of 20% sodium dodecyl sulfate were added to each well. The wells were stirred with a micropipette tip to remove amoebae from the bottom, and the mixture was added to 2 ml of ice-cold 10% TCA. The TCA precipitate was collected on glass fiber filters, washed with 10 ml of ice-cold 7% TCA, and measured for radioactivity as described above.

Estimation of the membrane potential. The membrane electrical potential (ΔΨ) of B. megaterium GR10 was determined by measuring the distribution of 3H-tetraphenylphosphonium ([3H]TPP+) across cell membranes (14, 15). [3H]TPP+ was added (6 μM, final concentration) to exponential-phase cells (400 to 500 μg [dry weight]/ml) incubated at 28°C under agitation. After 15 min of incubation, amoebicin m4-A (10 μg/ml) or Tris-HCl buffer was added. Triplicate portions (100 μl each) were removed at desired intervals,
FIG. 1. Effects of different concentrations of amoebicin m4-A on exponential-phase cultures of B. megaterium GR10 growing in BHI broth. The optical density at 620 nm (OD620) (A) and the number of viable cells (B) were measured for controls (○) and cultures incubated with 5 (□) or 10 (△) μg of purified amoebicin per ml. Arrowheads indicate the time of amoebicin addition.

filtered through Whatman GF/C filters, and washed twice with 3 ml of buffer. Aliquots were treated with 10% butanol to make corrections for nonspecific binding of [3H]TPP+. Gramicidin D (5 μg/ml, final concentration) was added as a positive control (13). The membrane electrical potential was calculated from the equation Δψ = -(RT/F)ln([TPP+in]/[TPP+out]), where R is the gas constant, F is Faraday’s constant, and T is the absolute temperature. An intracellular volume of 4.0 μl/mg of cells was calculated by the inulin exclusion method (17).

Preparation of freeze-thaw vesicles. l-α-Phosphatidylcholine from soy beans (type IV-S; Sigma) was homogenized (5 mg) in 2 ml of 20 mM sodium phosphate buffer (pH 7.2) by sonication. Vesicles were prepared in the presence of the labeled precursors 86Rb+ (2.72 μCi/mg), [5,6-3H]uridine (48 Ci/mmol), [4,5,6-3H]leucine (120 Ci/mmol), and [carboxyl-14C]dextran (0.6 mCi/g; M0,000,000 to 70,000) at final concentrations of 0.5, 5, 3, and 4 μCi/ml, respectively, by a procedure described previously (8). Triplicate portions (100 μl) of the vesicle suspensions were filtered through Millipore GSTF 02500 filters and washed with 2 ml of 100 mM lithium chloride. The radioactivity retained in the filters was measured as described above.

Electron microscopy of vesicles. Samples of liquid vesicles obtained by sonication as described above were treated with amoebicin for various periods. The vesicles were stained with 2% uranyl acetate as described previously (8) and observed under a Zeiss 902 transmission electron microscope.

RESULTS

Biological activity of amoebicin m4-A against B. megaterium GR10. Addition of amoebicin m4-A to exponentially growing cultures of B. megaterium GR10 caused a decrease in turbidity due to cell lysis (Fig. 1A). Simultaneously, the number of viable cells decreased rapidly after amoebicin addition (Fig. 1B). Both the bactericidal and the bacteriolytic effects were proportional to the amount of amoebicin added. Incorporation of radioactive precursors of macromolecular synthesis (protein, DNA, and RNA) into TCA-precipitable material was rapidly inhibited by amoebicin m4-A (Fig. 2A, B, and C, respectively), suggesting that the cytoplasmic membrane may be the primary site of action. To corroborate this hypothesis, we tested the effect of amoebicin on the membrane electrical potential of B. megaterium GR10 by measuring the distribution of the lipophilic cation [3H]TPP+. The Δψ decreased drastically after 10 min of incubation with amoebicin m4-A (Fig. 3). This effect was similar to that of the ionophore gramicidin D (Fig. 3).

Effect of amoebicin m4-A on the incorporation of labeled precursors by N. fowleri HB-1. The effects of purified amoebicin m4-A on the incorporation of precursors of macromolecular synthesis by N. fowleri HB-1 are shown in Fig. 4. Addition of amoebicin m4-A rapidly inhibited incorporation of labeled uridine and thymidine, and, furthermore, the cells lost most of the radioactive material previously incorporated.

Effect on liposomes. Addition of amoebicin (5 μg/ml, final concentration) to liposomes loaded with 86Rb+, [5,6-3H]uridine, or [carboxyl-14C]dextran caused depletion of all three radioactive markers within 5 min (Fig. 5A, B, and C, respectively).
electron microscope showed that incubation with amoebicin for 5 min caused formation of small holes in the liposome membrane, and 10 min of treatment induced complete lysis (data not shown).

**DISCUSSION**

The initial observation that cells of *N. fowleri* were lysed by partially and completely purified preparations of amoebicin from *B. licheniformis* M-4 suggested that the primary site of action of these antibiotics is the cell membrane (5, 16). In order to corroborate these results, we have studied the effects of amoebicin m4-A (the main antibiotic found in extracts of *B. licheniformis* M-4) on *B. megaterium* GR10, amoebae, and artificial membranes (asolecin vesicles). Inhibition of the incorporation of different radioactive precursors into macromolecules and depletion of the membrane electrical potential by this amoebicin are strong indications that the cytoplasmic membrane is the primary site of action. Furthermore, amoebicin m4-A not only inhibited incorporation of radioactive markers by *N. fowleri* HB-1 but also caused depletion of previously incorporated precursors. These results are consistent with the lytic effect of amoebicin m4-A occurring within the first hour of amoebicin addition (5, 16). Also, amoebicin m4-A is outstanding in its capacity to induce concentration-dependent permeation of liposomes in the absence of any induced membrane potential.

The lytic activity of amoebicin m4-A against *B. megaterium* GR10 resembles that of gramicidin D (13). Bacterial lysis by agents that interact with the cytoplasmic membrane has been explained as a secondary effect of depletion of either membrane electrical potential or pH gradient, which would be essential for control of autolysins (13). This is also the case for other peptide antibiotics affecting the cell membrane, such as peptide AS-48 from *Enterococcus faecalis* (8). This antibiotic also dissipates the membrane electrical potential of sensitive bacteria and causes lysis of liposomes in the absence of membrane potential but is not active against amoebae or fungi. Permeation of liposomes has also been reported for other peptide antibiotics, such as nisin (10) and colistin (4). The observation that different membrane-damaging peptide antibiotics show selective spectra of inhibition suggests that both cell wall structure and membrane composition are main factors in determining the sensitivity of microorganisms to antibiotics acting on the cell membrane. The selectivity of amoebicin m4-A against species of *Naegleria* but not against *Acanthamoeba* species was discussed in previous reports (5, 9) and is in accordance with these observations.

The fact that amoebicin m4-A is not toxic to the cell lines HeLa and MDCK (data not shown) and its high antiamoebic activity and water solubility (16) are promising features for further studies both in vitro and in vivo of its effectiveness against primary amoebic meningoencephalitis.

**REFERENCES**

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