Effect of trichothecenes on growth and intracellular pool size of *Mycoplasma gallisepticum*

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The mycotoxin T-2 inhibited the growth of *Mycoplasma gallisepticum*. The growth inhibition was most pronounced with the hydrophobic derivatives T-2 acetate and very little with the hydrophilic T-2 tetraol. The toxin had no effect on the biosynthesis of either protein, DNA, RNA or complex lipids but markedly reduced the intracellular pool size of soluble low molecular mass precursors. It seems that T-2 acetate, by virtue of its hydrophobic nature, may accumulate within the lipid backbone affecting the permeability properties of the cell membrane.

T-2 toxin Mycoplasma gallisepticum *Mycoplasma membrane* *Trichothecen* *Mycotoxin* *Intracytoplasmic pool*

1. INTRODUCTION

Trichothecenes are a group of mycotoxins widely distributed in nature and associated with tremendous damage to humans and farm animals [1]. Very little is known, however, regarding the mode of action of these toxins. In eucaryotes, trichothecenes were shown to inhibit protein synthesis [2]. Recent reports suggested that these toxins are active also at the cell membrane level. Thus, trichothecenes were shown to inhibit platelet aggregation [3], induce hemolysis of human red blood cells [4] and inhibit phagocytosis by polymorphonuclear cells [5]. To obtain further insight on the effect of trichothecenes on cytoplasmic membranes we investigated the effect of these toxins on mycoplasmas. These procaryotes have no cell wall or intracellular membrane structure and their cytoplasmic membrane can be easily isolated [6]. The ability to introduce controlled changes in the membrane composition of these organisms have been used intensively in a wide variety of membrane studies [7,8] and might enable a thorough investigation on the association of the trichothecenes with membrane components. The results of the study presented show that trichothecenes, mainly the hydrophobic derivatives, had a marked effect on the growth rate of *Mycoplasma gallisepticum* although protein synthesis was not affected. The toxins markedly reduced the intracellular pool size of low molecular mass precursors of protein RNA or DNA. The possibility that the effect on the pool size is caused by damage to the permeability properties of the membrane is discussed.

2. MATERIALS AND METHODS

2.1. *Trichothecene preparations*

T-2 toxin [3α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-en], was isolated from extracts of *Fusarium sporotrichioides* as in [9]. The derivatives HT-2, T-2 Tetraol and T-2 acetate were synthesized by us [10]. The macrocyclic trichothecenes Roridin and Verucarrin were kindly provided by Dr Y. Behrend, Makor Chemicals, Israel. The toxins were dissolved in dimethylsulfoxide (DMSO) and added to the growth medium. The final concentration of DMSO in the growth medium did not exceed 0.1%.

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2.2. *Organisms and growth conditions*

Mycoplasma gallisepticum was grown in Edward medium [6] containing 4% horse serum. To label membrane lipids, the medium was supplemented with 0.1 μCi/ml of [9,10-^{3}H]oleate (500 Ci/mol). To label cell protein the medium was supplemented with 1 μCi/ml of [4-^{3}H]phenylalanine (25 Ci/mmol). To label nucleic acids the medium was supplemented with either 1 μCi/ml of [*methyl*-^{3}H]thymidine (70 Ci/mmol) or with 1 μCi/ml of [5,6-^{3}H]uracil (50 Ci/mmol). All radioactive components were from Amersham Radiochemical Centre. The media were inoculated from an overnight culture at an inoculum level of 2–3% and the cultures were incubated for 16–28 h at 37°C. Growth was followed by measuring the absorbance of the culture at 640 nm. Most experiments were performed with cells harvested at the exponential phase of growth (16–20 h) by centrifugation at 12000 × *g* for 20 min. The cells were washed once and resuspended in cold 0.25 M NaCl solution. Membranes were prepared as in [6].

2.3. *Incorporation of radioactive precursors*

To determine the level of incorporation of the radioactive precursors into the washed cell preparations, 0.1 ml aliquots of the cell culture were filtered through a 0.45 μm pore size millipore filter. The filters were washed twice with 10 ml cold 0.25 M NaCl, dried and the radioactivity was determined in a Packard liquid scintillator using 3 ml toluene scintillation liquor. To determine the radioactivity incorporated into macromolecules, 0.1 ml aliquots of washed cells were added to 5 ml cold 10% trichloroacetic acid. The samples were incubated at 4°C for 60 min, filtered, washed twice with 10 ml of cold 10% trichloroacetic acid dried and counted.

2.4. *Analytical methods*

Cell water was measured using ^{3}H-labeled water (4.3 μCi/ml) and [^{14}C]inulin (0.4 μCi/ml) as in [11]. It was assumed that inulin (M_r 5000) did not penetrate into the cells and could be used to measure extracellular fluids in the cell pellet. The total water space minus the inulin space was taken as the intracellular water space. Protein in cell preparations was determined according to [12]. Proteins were analyzed on sodium dodecylsulfate–polyacrylamide gels as in [13]. Lipids were extracted from intact cells by the method in [14] and characterized as in [15].

3. RESULTS

Fig.1 shows that trichothecenes inhibited the growth of *M. gallisepticum*. The growth inhibition by the trichothecenes tested varied considerably. The lowest inhibitory effect was found with the more hydrophylic T-2 tetraol whereas the inhibition by T-2 acetate was the highest. All inhibition experiments were monitored after 18 h of incubation at 37°C. The growth response of *M. gallisepticum* in a medium with or without T-2 acetate is presented in fig.2. It is apparent that the extrapolated initial growth rate without the toxin was much faster than the rate measured in the presence of T-2 acetate. The absorbance at 640 nm of late exponential phase cultures was, however, very similar. To determine whether T-2 acetate affects macromolecule synthesis, *M. gallisepticum* cells were grown with radioactive precursors of phospholipids ([^{3}H]oleate), protein ([^{3}H]phenylal-

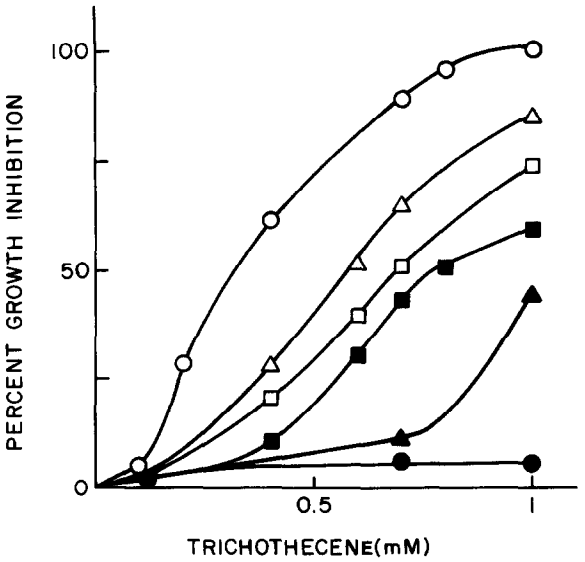

Fig.1. The effect of trichothecenes on the growth of *M. gallisepticum*. *M. gallisepticum* cells were grown with various concentrations of trichothecenes for 18 h. Growth was estimated from the absorbance of the culture at 640 nm. T-2 acetate (○); verucarrin (△); roridin (□); T-2 (■); HT-2 (▲); T-2 tetraol (●).

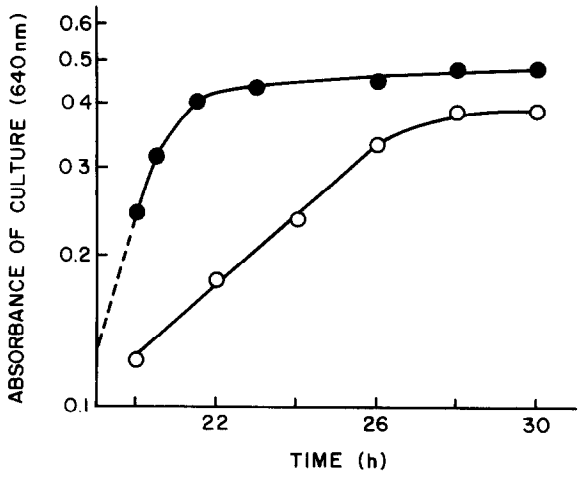

Fig.2. Growth curve of *M. gallisepticum* in the presence or absence of T-2 acetate. T-2 acetate (0.75 mM) was added to the growth medium at time 0. Culture treated with T-2 acetate (○); untreated control culture (●).

anine), RNA ([^{3}H]uracil) or DNA ([^{3}H]thymidine). The incorporation of radioactive oleate into T-2 acetate treated and untreated cells was almost the same (table 1). However, the incorporation of radioactive phenylalanine, uracil or thymidine into control cells was about 25–30% higher than in cells grown with T-2 acetate. Extraction of washed phenylalanine-labelled, uracil-labelled or thymidine-labelled cells with 10% cold trichloroacetic acid released 20–30% of the total radioactivity from the control cells, but only up to 5% of the radioactivity from cells grown with T-2 acetate. As a result the levels of residual radioactivity in the trichloroacetic acid extracted control and T-2 acetate treated cells were almost the same. Analysis of membranes from cells grown with or without T-2 acetate revealed almost identical phospholipid and cholesterol contents, identical polypeptide patterns (not shown) and a very similar phospholipid composition comprising of sphingomyelin, phosphatidylcholine and phosphatidylglycerol (21, 34 and 40% of total phospholipids, respectively). The intracellular cell volume of untreated and T-2 acetate treated cells was almost identical (2.1 and 2.3 μl/mg protein, respectively).

4. DISCUSSION

Our findings that the growth of *M. gallisepticum* was inhibited by trichothecenes indicates that these compounds, known to be highly cytotoxic to eucaryotic cells [1,16] inhibit also the growth of procaryotes. The growth inhibition was much more pronounced with the hydrophobic derivatives, mainly with T-2 acetate, though this compound is 100 times less toxic than T-2 for HeLa cells [16]. The higher inhibitory activity of the

Table 1

The effect of T-2 acetate on the incorporation of radioactive precursors into *M. gallisepticum* cells

^{3}H-labeled precursor	Radioactivity in cells (cpm/mg cell protein)			
	Cells grown without T-2 acetate		Cells grown with T-2 acetate	
	Intact	Trichloroacetic acid extracted	Intact	Trichloroacetic acid extracted
Oleic acid	307900	297800	302000	294000
Phenylalanine	27000	18300	19400	18700
Uracil	134000	107400	95600	98000
Thymidine	560900	402600	388000	369800

The organisms were grown in a medium containing either [9,10-^{3}H]oleate, [4-^{3}H]phenylalanine, [5,6-^{3}H]uracil or [*methyl*-^{3}H]thymidine with or without T-2 acetate (150 μg/ml). The distribution of label and Trichloroacetic acid extraction were performed as in section 2

hydrophobic trichothecenes is due either to the fast penetration of the more hydrophobic derivatives or to their accumulation within the cell membrane to concentrations sufficient to exhibit the toxic effect. The mechanism by which T-2 acetate inhibits the growth of *M. gallisepticum* is not yet understood. In eucaryotes, trichothecenes are potent inhibitors of protein synthesis and to a lesser degree inhibit the biosynthesis of DNA and RNA [17–19]. In *M. gallisepticum*, however, the gross protein, DNA, RNA and complex lipid biosynthesis in the presence of T-2 acetate remained unchanged. Our observation that cells treated with T-2 acetate possess low levels of trichloroacetic acid extractable material suggests that T-2 acetate affects the intracellular pools of soluble low molecular mass precursors. However, the toxin has no effect on the free fatty acid pool that accumulates within the cell membrane. As the toxin affects precursor pools utilized for protein, DNA and RNA biosynthesis to about the same extent, it seems that its effect is non-specific, probably resulting from damage to the cell membrane. This concept was also suggested from electron paramagnetic resonance studies demonstrating a lower freedom of motion of spin-labelled phospholipids in membranes from T-2 acetate treated *M. gallisepticum* cells (P.J. Davis, unpublished).

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