***Mycoplasma penetrans* infection of Molt-3 lymphocytes induces changes in the lipid composition of host cells**

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The AIDS-associated *Mycoplasma penetrans* is capable of inducing its own uptake by non-phagocytic cells. The ability of *M. penetrans* to both adhere to and invade Molt-3 lymphocytes was markedly increased in the presence of polyethylene glycol 8000 (PEG). The effect of PEG was more pronounced in the more alkaline pH range, where the binding kinetics were much faster and almost unaffected by temperature (4–37 °C). Incubation of [¹⁴C]oleic-acid-labelled Molt-3 cells with viable *M. penetrans* resulted in a substantial release of radioactive fatty acids, whereas treating the host cells with heat-inactivated mycoplasmas, isolated *M. penetrans* membrane preparations, or *M. penetrans* growth medium, had no effect. Total lipid analysis of Molt-3 lymphocytes infected by *M. penetrans* revealed an augmented level of the neutral lipid fraction that was associated with a decrease in the relative amounts of polar lipids, mainly a decrease in the amount of phosphatidylserine and diphosphatidylglycerol. Analysis of the neutral lipid fraction in the infected Molt-3 cells revealed a fivefold increase in the relative amount of diacylglycerol and a marked increase in the free fatty acid (FFA) fraction. The profile of the FFAs released was dominated by a relatively high concentration of the polyunsaturated fatty acid docosahexaenoic acid. The release of lipid intermediates suggests that the degradation of Molt-3 cell phospholipids induced by *M. penetrans* may initiate a signal transmission cascade in the host cell.

Keywords: mollicutes, *Mycoplasma penetrans*, phospholipase, membrane lipids, diacylglycerol

INTRODUCTION

Mycoplasmas (class *Mollicutes*) are simple self-replicating prokaryotes widely distributed in nature as parasites and pathogens of animals, insects and plants. Almost all pathogenic mycoplasmas are bound to the cell surface of the host cells without being able to invade them (Razin & Jacobs, 1992). However, *Mycoplasma penetrans*, recently isolated from the urogenital tract of AIDS patients, was shown to have invasive properties

and an intracellular location in eukaryotic host cells (Lo, 1992; Lo *et al.*, 1993; Andreev *et al.*, 1995; Giron *et al.*, 1996). *M. penetrans* also has a profound synergistic effect on HIV-1 cytopathogenic activities in infected human lymphocyte cultures (Lo, 1992) and it has been suggested that this organism plays a role in the pathogenesis of AIDS (Lo, 1992; Brenner *et al.*, 1996).

Invasion is initiated by the binding of bacteria to the host-cell surface, followed by internalization (Falkow *et al.*, 1992). We recently showed that in *M. penetrans*-infected eukaryotic cells, protein kinase C (PKC) is translocated from the cytosol of the host cell to the plasma membrane (Borovsky *et al.*, 1998), indicating the activation of the enzyme by an agonist (Neyrolles *et al.*, 1996). The internalization of *M. penetrans* by non-phagocytic host cells is also dependent upon microfilament and/or microtubule functions, since the

Abbreviations: DAG, diacylglycerol; DHA, docosahexanoic acid; DPG, diphosphatidylglycerol; FFA, free fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, polyethylene glycol 8000; PG, phosphatidylglycerol; PI, phosphatidylinositol; PKC, protein kinase C; PLA₂, phospholipase A₂; PS, phosphatidylserine; SPM, sphingomyelin; TG, triglycerides.

internalization is prevented by cytochalasin D, which inhibits microfilament assembly (Andreev *et al.*, 1995; Baseman *et al.*, 1995; Giron *et al.*, 1996), or by vinblastine, which disorganizes microtubules (Andreev *et al.*, 1995; Borovsky *et al.*, 1998). The activation of PKC and the active involvement of the host cytoskeleton suggests that *M. penetrans* generates 'uptake signals' which are transduced between the mycoplasma and the host cell across the host-cell membrane (Wick *et al.*, 1991; Ruschkowsky *et al.*, 1992; Giron *et al.*, 1996). However, the nature of these signals and the mechanisms used to transduce them remain unknown.

We have recently shown that a 145 kDa host-cell protein is tyrosine phosphorylated as a result of binding and/or invasion of a host cell by *M. penetrans* (Andreev *et al.*, 1995) and the possibility that this protein represents a membrane-bound phospholipase has been suggested (Andreev *et al.*, 1995; Borovsky *et al.*, 1998).

The aim of this study was to investigate phospholipid degradation in Molt-3 lymphocytes infected by *M. penetrans* and to characterize the lipid intermediates released.

METHODS

Growth of mycoplasmas and tissue culture cells. *M. penetrans* was kindly provided by Dr S.-C. Lo (Armed Forces Institute of Pathology, Washington, DC, USA). The organisms were grown in modified Channock medium (Razin & Rottem, 1976), supplemented with 10% (v/v) heat-inactivated (56 °C for 30 min) horse serum (Biological Industries). *Mycoplasma capricolum* (strain California Kid) was grown in modified Edward medium (Razin & Rottem, 1976), supplemented with 4% (v/v) heat-inactivated horse serum. For labelling of *M. penetrans* membrane lipids, 0.02 µCi ml⁻¹ (740 Bq ml⁻¹) [9,10(n)-³H]palmitic acid (Amersham), dissolved in ethanol, was added to the growth medium. For labelling cell proteins, 5 µCi ml⁻¹ (185 kBq ml⁻¹) L-[³⁵S]methionine (Amersham) was added to the medium. The media were inoculated with 0.1–2.0% (v/v) of a frozen inoculum and incubated at 37 °C for 18–26 h. Growth was followed by measuring OD₆₄₀, and by following pH changes in the growth media. Cells were harvested by centrifugation at 12000 g for 15 min, washed once and suspended in A-buffer, consisting of 250 mM NaCl, 10 mM MgCl₂ and 10 mM Tris/HCl adjusted to pH 7.4.

The human lymphocyte cell line CD4⁺ Molt-3 was obtained from the American Type Culture Collection and maintained in RPMI 1640 medium, 20% (v/v) foetal calf serum (FCS), penicillin (100 µg ml⁻¹) and streptomycin (100 µg ml⁻¹). All medium components were obtained from Biological Industries. Molt-3 cells were labelled with [U-¹⁴C]palmitic acid (Amersham) [928 Ci mol⁻¹ (34 TBq mol⁻¹)], [U-¹⁴C]oleic acid (Amersham) [57.4 Ci mol⁻¹ (2.1 TBq mol⁻¹)] or [³²P]orthophosphate (Amersham) [3000 Ci mmol⁻¹ (111 TBq mol⁻¹)] which were added to the growth medium [0.1–0.2 µCi ml⁻¹ (3.7–7.4 kBq mol⁻¹)] 24 h before harvesting. The fatty acids were dissolved in ethanol and the final concentration of ethanol in the medium did not exceed 0.1%. Cells were used prior to passage 40 and grown and assayed at 37 °C, 5% CO₂ and 90% humidity. Molt-3 cells were inoculated into the medium at a 1:10 ratio and grown to a density of 1–1.2 × 10⁶ cells ml⁻¹ (about 72 h). Viability of Molt-3 cells was evaluated by the trypan blue exclusion.

Binding and invasion of *M. penetrans*. To determine the number of bound *M. penetrans* cells, we used *M. penetrans* cells metabolically labelled by [³H]palmitate or [³⁵S]methionine. When cells are grown with radioactive palmitic acid, over 95% of the radioactivity is incorporated into the phospholipid fraction in two *de novo* synthesized phospholipids, phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) (Salman & Rottem, 1995). The radioactive mycoplasmas were added to Molt-3 cells in RPMI 1640 medium at a m.o.i. of 100 mycoplasmas per target cell and incubated for various periods of time at 4 °C. The cell cultures were then washed twice with PBS to remove non-adherent bacteria and radioactivity was measured. Invasion of Molt-3 cells by *M. penetrans* was determined by the plating method (Andreev *et al.*, 1995). Molt-3 cells were harvested at a density of 10⁶ cells ml⁻¹, washed and resuspended in RPMI 1640 medium. Aliquots of 4 ml were then inoculated with *M. penetrans* at a m.o.i. of 100 in the presence or absence of polyethylene glycol 8000 (PEG) and incubated for 2 h at 37 °C. The cultures were then washed twice, resuspended in RPMI 1640 containing 400 µg gentamicin ml⁻¹ (Biological Industries) and 0.01% Triton X-100, and incubated for 2 h at 37 °C. Following incubation, the lymphocytes were plated onto solid mycoplasma medium. Each mycoplasma colony obtained by this method represents an infected host cell rather than a single intracellular mycoplasma cell (Shaw & Falkow, 1988).

Lipid analysis. Lipids were extracted from washed Molt-3 cells according to the method of Bligh & Dyer (1959). Lipid phosphorus was determined after digestion of the sample in ethanolic magnesium nitrate (Rottem & Markowitz, 1979). The lipids were separated by silicic acid column chromatography. The total lipid extract (200 mg) in 2 ml chloroform was loaded onto a silicic acid column that was eluted with 10 bed volumes of chloroform followed by 15 bed volumes of chloroform/methanol 1:1 (v/v). The fractions were evaporated to dryness under a stream of nitrogen, redissolved in chloroform and kept at –20 °C. The neutral lipid fraction, eluted from the column with chloroform, was separated by chromatography on silica gel G plates (Sigma) using the two-step development system (Rottem, 1980). The plates were first developed in benzene/diethyl ether/ethanol/acetic acid (50:40:2:0.2, by vol.), then dried and developed in the same direction in hexane/diethyl ether (94:6, v/v), (Rottem, 1983). The polar lipid fractions eluted from the column with chloroform/methanol were separated on silica gel HR plates and developed at 4 °C in chloroform/methanol/water (65:25:4, by vol.). Two-dimensional TLC of polar lipids was performed on silica gel HR plates: the plates were developed in the first direction in chloroform/methanol/28% ammonia (65:25:4, by vol.), and in the second direction in chloroform/methanol/water (65:25:4, by vol.). Polar lipid standards were run in both directions on the right sides of the TLC plates. Lipid spots were detected by iodine vapour, phospholipid spots by molybdate spray reagent, glycolipids by anthrone reagent and aminophospholipids by ninhydrin reagent (Rottem, 1983).

To quantify the phosphorus in the phospholipid spots resolved by TLC, the spots were scraped off the plate and digested with 0.2 ml ethanolic magnesium nitrate solution in the presence of silica gel. To measure the radioactivity of the lipid spots, the spots were scraped off the plate into scintillation vials containing 5 ml Lumax scintillation liquor and radioactivity was measured in a Packard Tri-Carb scintillation spectrometer and expressed as d.p.m. Alternatively, the TLC plates were exposed to imaging plates (Fuji Photo Film) for 6 h and then processed, using the Fujix BAS 1000 (Fuji) radiation

image analysis system and the radioactivity in each lipid spot was determined.

Fatty acid analysis. Fatty acid methyl esters were prepared by heating the lipid samples in 14% boron trifluoride in methanol at 72 °C for 15 min. The resulting methyl esters were extracted twice with hexane and subjected to GLC in a Trakor 540 gas-liquid chromatograph equipped with a polar column (100 × 0.2 cm, packed with 10% SP 2330 on 100/120 mesh Chromosorb W, Supelco). Fatty acids were identified by their retention times relative to those obtained with standard mixtures of fatty acid methyl esters (Rottem, 1983). Alternatively, fatty acid methyl esters were separated on silica gel G plates impregnated with 15% (w/v) AgNO₃ for separation of fatty acids with different degrees of unsaturation. For separation of fatty acids with 0–2 double bonds, a mixture of hexane/diethyl ether (9:1, v/v) was used, and for separation of fatty acids with 3–6 double bonds, a mixture of hexane/diethyl ether (2:3, v/v) was used (Christie, 1989).

RESULTS

Effect of PEG on binding and internalization of *M. penetrans*

It has been previously shown that *M. penetrans* adheres to cultured human cells in a time-dependent manner but the binding rates observed were very slow, with maximal binding obtained after 6–12 h incubation (Giron *et al.*, 1996). To check the possibility that the slow binding rate was due to a water layer adjacent to the surface polysaccharide, recently described in *M. penetrans* (Neyrolles *et al.*, 1996), we used PEG to perturb surface water structure and allow better contact between cells (Lucy & Ahkong, 1989). Fig. 1 shows that when ³H-labelled *M. penetrans* was incubated with Molt-3 cells for 2 h at 37 °C the level of adherence was markedly affected by PEG. The process was concentration de-

Fig. 1. Effect of PEG on the binding of ³H-labelled *M. penetrans* to Molt-3 cells at various pHs. Labelled *M. penetrans* were incubated with Molt-3 cells (m.o.i. = 100) and various PEG concentrations for 2 h at 4 °C. Binding was carried out at pH 5.0 (Δ), pH 7.0 (□) or pH 9.0 (○). Data represent means ± SD from three independent experiments.

Fig. 2. Effect of temperature and PEG on the binding of ³H-labelled *M. penetrans* to Molt-3 cells. Labelled *M. penetrans* were incubated with Molt-3 cells at a m.o.i. = 100 for various periods of time with (filled symbols) or without (open symbols) 8% PEG. Binding was assayed at 4 °C (□, ■) or 37 °C (Δ, ▲). Results presented are means ± SD from three independent experiments.

pendent, with marked enhancement of binding at 8–12% PEG. The effect of PEG was most pronounced at pH 7.0 and 9.0, and less pronounced at pH 5.0. Fig. 2 shows the profile of binding of *M. penetrans* to Molt-3 cells in the presence and absence of PEG over time. In the presence of 8% PEG, the binding kinetics were much faster and almost unaffected by temperature (4–37 °C), whereas in the absence of PEG, the levels of binding at 37 °C were higher than those seen at 4 °C. PEG also had a marked effect on the internalization of *M. penetrans* by Molt-3 cells (Table 1). Internalization was studied by the plating method, where gentamicin resistance in the presence of 0.01% Triton X-100 is used to determine intracellular mycoplasmas (Andreev *et al.*, 1995). In the presence of 8% PEG the number of Molt-3 cells invaded by *M. penetrans* was markedly higher than the number of invaded cells in the absence of PEG (Table 1). Therefore, PEG (8% v/v) was added to all subsequent experiments. Neither binding nor internalization was affected by inhibiting protein synthesis in *M. penetrans* by pretreatment with chloramphenicol (50–100 µg ml⁻¹) for 2 h at 37 °C. However, internalization was almost completely inhibited (93 ± 5% inhibition; mean ± SD, *n* = 3) by cytochalasin D (5 µg ml⁻¹).

Effect of *M. penetrans* on the release of free fatty acids from Molt-3 cells

Treatment of [¹⁴C]oleic-acid-labelled Molt-3 cells with pancreatic phospholipase A₂ (PLA₂) resulted in the release of 86 ± 6% (mean ± SD, *n* = 3) of the incorporated radioactivity as free fatty acids (FFA) compared with a release of about 21% obtained when [¹⁴C]palmitic acid-labelled Molt-3 cells were treated with PLA₂, suggesting that most of the oleic acid added

Table 1. Effect of PEG on binding to and invasion of Molt-3 cells by *M. penetrans*

Molt-3 cells were infected with palmitic-acid-labelled *M. penetrans* at 4 °C or 37 °C for 2 h. Binding was calculated from the residual radioactivity associated with the Molt-3 cells incubated with the mycoplasmas at 4 °C. The data presented are means $\pm$ SD of three different experiments. PFA, paraformaldehyde.

Condition	Molt-3 cells treated with	Bound <i>M. penetrans</i> (c.p.m. per 10 ⁶ Molt-3 cells)	Internalized <i>M. penetrans</i> (c.f.u. per 10 ⁶ Molt-3 cells)
Without PEG	None	1390 $\pm$ 400	5500 $\pm$ 2000
	PFA (4%)	1460 $\pm$ 450	2000 $\pm$ 1000
With PEG (8%)	None	7460 $\pm$ 680	17000 $\pm$ 2000
	PFA (4%)	6800 $\pm$ 720	2500 $\pm$ 1000

Table 2. Effect of mycoplasmas on the release of FFA from Molt-3 cells

[¹⁴C]Oleate-labelled Molt-3 cells were incubated for 18 h with various stimulants. The concentration of stimulants other than viable mycoplasmas was standardized to m.o.i. = 100. The data presented are means $\pm$ SD of three different experiments.

Molt-3 cells treated with	Radioactivity in FFA fraction (%)	Viability of Molt-3 cells (%)
None	<0.2	90 $\pm$ 5
<i>M. penetrans</i> cells	8.3 $\pm$ 2.6	70 $\pm$ 8
Heat-inactivated cells	<0.2	83 $\pm$ 10
Isolated membranes	<0.2	86 $\pm$ 7
Soluble fraction	<0.2	89 $\pm$ 9
Lipid extract	<0.2	94 $\pm$ 4
<i>M. capricolum</i> cells	<0.2	64 $\pm$ 8
Medium*	<0.2	92 $\pm$ 5

*The medium was obtained after harvesting *M. penetrans* cells and was added to the RPMI medium to a final concentration of 10%.

to the medium was incorporated as an unsaturated fatty acyl into position 2 of the glycerol. To verify this observation, total lipids of palmitate- and oleate-labelled Molt-3 cells were transmethylated, and the fatty acid methyl esters were separated by argentation TLC, according to the number of double bonds (Christie, 1990). Autoradiography of the plates revealed that following labelling with oleic acid, most of the radioactivity (over 90%) was recovered in the mono-unsaturated band, suggesting that the majority of the oleic acid incorporated from the medium was not metabolized. Only 2.8% of the radioactivity was detected in the saturated fatty acid band, and 5.4% in the polyunsaturated fatty acid band. When the cells were labelled with palmitic acid, about 75% of the total radioactivity was recovered in the saturated fatty acid

band, whereas 15% was detected in the mono-unsaturated fraction. [¹⁴C]Oleic-acid-labelled Molt-3 cells were, therefore, used in subsequent experiments. Incubation of *M. penetrans* with [¹⁴C]oleic-acid-labelled Molt-3 cells at a m.o.i. of 100 resulted in a substantial release of radioactive FFA (Table 2), compared with the extremely low level (approx. 0.2%) of FFA in a control of untreated Molt-3 cells. FFA release was observed only with viable *M. penetrans*, whereas heat-inactivated mycoplasmas (65 °C, 10 min), isolated *M. penetrans* membrane preparations, and the soluble fraction derived from *M. penetrans* after disrupting the mycoplasmas by ultrasonic treatment, had no effect. Table 2 also shows that the viability of Molt-3 cells incubated with *M. penetrans* was moderately affected (a decrease of 20–30%). To determine whether the fatty acid release was due to the death of host cells rather than to the invasion process, a control experiment was performed with the non-invasive *M. capricolum* that, under identical experimental conditions, was found to induce a more pronounced cell death of Molt-3. However, this organism did not cause appreciable FFA release (Table 2). FFA release was also observed when native Molt-3 cells were infected with *M. penetrans* in the presence of chloramphenicol but not after *M. penetrans* infection of cytochalasin D-treated Molt-3 cells (Table 3). The decrease in viability of cytochalasin D-treated cells incubated with *M. penetrans* was identical to that of infected native Molt-3 cells (Table 3). As can be seen in Fig. 3, released FFA were first detected after 4 h incubation, with maximal accumulation observed at 18 h incubation.

Changes in the polar and neutral lipid composition of Molt-3 cells incubated with *M. penetrans*

The polar lipid composition of Molt-3 cells incubated with or without *M. penetrans* was analysed by two-dimensional TLC of [³²P]orthophosphate-labelled Molt-3 cells followed by autoradiography. A pronounced decrease in the amount of phosphatidylserine (PS) and a reduced concentration of DPG were detected in cells

Table 3. Release of FFA from Molt-3 cells incubated with *M. penetrans*

[¹⁴C]Oleate-labelled Molt-3 cells were incubated for 12 h with native or with chloramphenicol (100 µg ml⁻¹) treated *M. penetrans* at a m.o.i. of 100. The data presented are means ± SD of three different experiments.

Molt-3 cells treated with	<i>M. penetrans</i> preparation	Radioactivity in FFA fraction (%)	Viability of Molt-3 cells (%)
Paraformaldehyde (4%)	Native	<0.2	0
Cytochalasin D	Native	<0.2	80 ± 5.0
Quinacrine (100 µM)	Native	<0.2	76 ± 5.0
Mepacrine (100 µM)	Native	<0.2	80 ± 5.0
None	Native	7.8 ± 2.5	75 ± 5.0
None	Chloramphenicol treated	6.6 ± 2.0	80 ± 5.0

Fig. 3. Kinetics of FFA release. Molt-3 cells were incubated for the designated periods of time with (○) or without (●) *M. penetrans* at a m.o.i. of 100. Lipids were extracted and the radioactivity in the FFA fraction was determined as described in Methods. Results are expressed as the mean radioactivity in the FFA fraction in triplicate experiments ± SD.

incubated with *M. penetrans* (Table 4). As the total amount of polar lipids in the cells incubated with *M. penetrans* was lower than that in control cells incubated without the mycoplasma (a polar to neutral lipid ratio of 2.5, compared with 4.9 in the control cells), an increase in the relative amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was also recorded.

Analysis of the neutral lipid fraction of cells is presented in Table 5. A fivefold increase in the relative amount of diacylglycerol (DAG) and a marked increase in FFA were observed in Molt-3 cells infected with *M. penetrans*. The increase in the total neutral lipids was associated with a decrease in the relative amounts of the polar lipids.

Identification of the fatty acids released from the Molt-3 lipids after interaction with *M. penetrans*

The fatty acid composition of the total lipid fraction of Molt-3 cells and of the FFA fraction released from Molt-3 cells upon incubation with *M. penetrans* is shown in Table 6. The main saturated fatty acids in the total lipid fraction were palmitic (16:0), stearic (18:0) and myristic acid (14:0); the unsaturated fatty acids consisted primarily of oleic (18:1) palmitoleic (16:1), arachidonic (20:4) and docosahexanoic acid (DHA, 22:6). The fatty acid profile of the FFA fraction obtained from Molt-3 cells incubated with *M. penetrans* revealed a higher level of unsaturated fatty acids, with an unsaturated to saturated fatty acid ratio of 1.21, compared with a ratio of 0.92 in the total lipid fraction of the infected cells and 0.75 in the total lipid fraction of uninfected control cells. The fatty acid profile of the FFA fraction was dominated by high concentrations of the polyunsaturated fatty acid DHA. The levels of DHA were always three- to fourfold higher than those of the arachidonic acid released. The PLA₂ inhibitors quinacrine and mepacrine did not affect the release of fatty acids (Table 3).

DISCUSSION

The prominent effect of *M. penetrans* in enhancing HIV-1 cytopathogenicity of human lymphocyte cultures (Lo, 1992) prompted us to investigate the effects, at the molecular level, of *M. penetrans* on a human CD4⁺ lymphocyte cell line. We found that PEG markedly increased the ability of the mycoplasma to both adhere to and invade Molt-3 lymphocytes, as judged by the resistance to extracellular gentamicin (Andreev *et al.*, 1995). Many investigators have recognized the ability of PEG to alter the physical state of bulk water adjacent to the cell membrane surface (Lucy & Ahkong, 1989; Nir *et al.*, 1986). At high concentrations (≥20%) PEG was found to destabilize the lipid backbone (Lucy & Ahkong, 1989). We observed enhanced binding of *M. penetrans* to Molt-3 cells at a relatively low PEG concentration (8%) that causes virtually no change in

Table 4. Phospholipid composition of ^{32}P -labelled Molt-3 cells incubated with or without *M. penetrans*

Values are means $\pm$ SD of three different experiments.

Phospholipid	Radioactivity (%) in phospholipids extracted from Molt-3 cells incubated	
	Without <i>M. penetrans</i>	With <i>M. penetrans</i>
Sphingomyelin	1.2 ± 0.2	1.5 ± 0.2
Phosphatidylserine	12.3 ± 1.6	3.2 ± 0.6
Phosphatidic acid	0.3 ± 0.1	< 0.1
Phosphatidylcholine	48.8 ± 4.0	53.9 ± 4.5
Phosphatidylethanolamine	29.1 ± 3.0	31.7 ± 3.0
Diphosphatidylglycerol	6.3 ± 0.8	2.8 ± 0.5
Phosphatidylglycerol	< 0.1	< 0.1

Table 5. Neutral lipid composition of [^{14}C]oleate-labelled Molt-3 cells incubated with or without *M. penetrans*

[^{14}C]Oleate-labelled Molt-3 cells were incubated with or without *M. penetrans* (m.o.i. = 100) for 18 h. The values presented are means $\pm$ SD of three different experiments.

Neutral lipids	Radioactivity in neutral lipids (%)		
	Molt-3 incubated without <i>M. penetrans</i>		Molt-3 incubated with <i>M. penetrans</i>
	(0 h)	(18 h)	(18 h)
Monoglycerides	< 0.1	< 0.1	< 0.1
Diglycerides	0.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.3
Triglycerides	15.0 ± 4.0	15.5 ± 3.2	18.7 ± 2.5
Free fatty acids	0.2 ± 0.1	1.3 ± 1.0	8.0 ± 2.0
Total neutral lipids	15.5 ± 4.2	17.2 ± 4.3	27.9 ± 4.8

Table 6. Fatty acid composition of the total lipid and FFA fractions released from Molt-3 cells infected with *M. penetrans*

The fatty acid methyl esters were identified on the basis of retention time compared with that of commercial standards. The data shown are the means of two experiments.

Molt-3 cell preparation	Lipid fraction*	Fatty acid† (mol%)												
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:5	22:5	22:6
Uninfected	TL	13.0	25.5	3.8	19.5	27.0	1.0	1.5	< 0.5	< 0.5	6.0	1.5	< 0.5	2.5
Infected	TL	12.5	23.1	5.1	16.1	26.0	1.2	1.2	0.6	0.8	5.3	0.8	0.8	5.9
	FFA	10.5	20.3	7.5	14.6	27.5	—	—	—	—	3.2	—	—	16.7

* TL, total lipids; FFA, free fatty acids.

† The first number indicates the number of carbon atoms and the second number, the number of double bonds.

the freedom of motion of membrane phospholipids (M. Salman & S. Rottem, unpublished data). Hence, it is likely that the perturbations in the surface water structure induced by PEG sufficed to enhance the binding of the mycoplasma. Uptake of *M. penetrans* by Molt-3 cells was not dependent on protein synthesis, as pre-treatment of *M. penetrans* with chloramphenicol affected neither their binding nor their internalization by the host cells.

Infection of Molt-3 lymphocytes by *M. penetrans* resulted in a substantial release of fatty acids, suggesting changes in the lipid turnover of the host cells. By contrast, very low concentrations of fatty acids were released when Molt-3 cells were incubated with *M. capricolum*, although the latter exhibited a higher cytotoxicity toward lymphocytes than the *M. penetrans* preparation. Furthermore, fatty acid release was not observed when heat-inactivated intact *M. penetrans* cells or isolated *M. penetrans* membranes were used or after *M. penetrans* infection of cytochalasin D-treated Molt-3 cells. These observations suggest that fatty acids are being released due to the invasion of Molt-3 cells by *M. penetrans* rather than to Molt-3 cell death or to toxic effects of *M. penetrans*. The finding that accumulation of fatty acids was detected only after 6 h of incubation, and reached a high level only at 18 h, suggests that the release is a slow process. Our fatty acid analysis revealed that both saturated and unsaturated free fatty acids were released. The unsaturated ones were predominantly very long polyunsaturated DHA (22:6), which constituted about 17% of the total FFA released, although in the total lipid fraction, DHA constitutes some 6%. DHA is widely present in human tissues (Katan *et al.*, 1997) but scant information is available on its physiological role.

The findings that the major neutral lipid fraction present in Molt-3 cells infected by *M. penetrans* was the FFA fraction that contained almost equimolar amounts of saturated and unsaturated fatty acids suggests that they are formed by the activity of lipases and/or non-specific esterases rather than by PLA₂, which liberates principally unsaturated FFA (Rottem, 1980). This notion was further supported by our inability to prevent FFA release by PLA₂ inhibitors (mepacrine or quinacrine). It is also unlikely that phospholipase A₁, recently characterized in *M. penetrans* membranes (Salman & Rottem, 1995), is responsible for the fatty acid release because it attacks the sn-1 position of phospholipids, where most of the fatty acids are saturated ones. It is possible that in our experiments the source of the released unsaturated fatty acids was the DAG accumulated as a result of phospholipase C activity (Shibata *et al.*, 1995), which could have been hydrolysed by the sequential action of 1-diacylglycerol lipase and 2-monoacylglycerol lipase (Balsinde *et al.*, 1991).

Are other lipid intermediates rather than FFA being released and what is the source of the FFA released in the infected lymphocytes? Analysis of the neutral lipid fraction from Molt-3 cells incubated with *M. penetrans*

revealed an increase in the amount of DAG, a well-established second messenger released apparently due to the activation of phospholipase C (Liscowitch, 1992). The finding of a pronounced reduction in the amount of phosphatidylserine (PS) and DPG suggests that these lipids are the major phospholipids hydrolysed. We recently showed that the interaction of *M. penetrans* with HeLa cells leads to the activation of PKC, which takes part in cellular responses to various agonists (Borovsky *et al.*, 1998). A similar activation has been observed when Molt-3 cells were infected with *M. penetrans* (M. Tarshis & S. Rottem, unpublished data). PKC activation can be triggered by an increased concentration of membrane DAG, resulting from agonist-induced hydrolysis of inositol phospholipids by phospholipase C (Nishizuka, 1992; Wick *et al.*, 1991). The DAG thus generated is frequently followed by a more sustained increase in DAG, due to the hydrolysis of other membrane phospholipids by phospholipase D in an agonist-dependent manner, culminating in the formation of phosphatidic acid that is then converted to DAG by a phosphatidic acid phosphomonoesterase (Nishizuka, 1992). Several *cis*-unsaturated fatty acids, including oleic, linoleic, linolenic, arachidonic and docosahexanoic acid, also enhance the DAG-dependent activation of PKC, allowing PKC to exhibit nearly full activity (Liscowitch, 1992; Nishizuka, 1992). In our study, the level of DAG produced in *M. penetrans*-stimulated Molt-3 cells was low, but almost five times higher than that in unstimulated control cells. The low DAG level may be accounted for by the high levels of DAG lipase and non-specific esterases present in T-lymphocytes (Nishizuka, 1992).

In conclusion, we have provided evidence that infection of lymphocytes by *M. penetrans* is associated with the release of lipid intermediates that may function in signal transduction across the host-cell membrane. The role of these signals in the penetration, survival and proliferation of the mycoplasma within the lymphocytes, as well as the involvement of the lipid intermediates in the pathobiological alterations taking place in the host cells, merit further investigation.

ACKNOWLEDGEMENTS

We would like to thank J. Andreev and A. Katzenel for their invaluable assistance. This study was supported by a grant from the Israel Ministry of Health.

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Received 13 January 1998; revised 22 May 1998; accepted 27 August 1998.