The AIDS-associated Mycoplasma penetrans is capable of inducing its own uptake by non-phagocytic cells. This study investigated the invasion of HeLa cells and its consequences by confocal laser scanning microscopy. Invasion was dependent on the duration of infection and temperature, diminished by inhibiting microfilament assembly with cytochalasin D and almost completely abolished by disorganising microtubules with vinblastine or taxol. After a short infection period (≤ 20 min), pronounced activation of protein kinase C was detected in host cells, whereas prolonged infection resulted in intensive vacuolation of the host cells and a pronounced increment in intracellular organic peroxide levels. A marked decrease in the extent of vacuolation was observed when peroxide accumulation was partially prevented by α-tocopherol. The possibility that M. penetrans entry into HeLa cells involves the activation of protein kinases and the recruitment of cytoskeleton components is discussed.

**Introduction**

Mycoplasmas (class Mollicutes) are the smallest and simplest self-replicating prokaryotes. They are widely distributed in nature as parasites and pathogens of both plants and animals. It has been generally accepted that pathogenic mycoplasmas are bound to the cell surface of the host cells without being able to invade them [1]. Nonetheless, intracellular location of Mycoplasma fermentans and M. hominis in cultured cells has been demonstrated by immunochromy and electron microscopy [2] and M. penetrans, recently isolated from the urogenital tract of AIDS patients, was shown to have invasive properties and intracellular location in eukaryotic host cells [3, 4].

Many pathogenic bacteria are capable of invading non-phagocytic host cells. Invasion by the pathogen is initiated by the binding of the bacteria to the host cell surface, followed by internalisation [5]. In studying cell invasion it is necessary to discriminate between external and internal bacteria [5, 6]. Among the techniques used to determine internalisation are optical and transmission electron microscopy, conventional fluorescence microscopy, antibiotic treatment of non-ingested cells and flow cytometry [5, 7]. Confocal laser scanning microscopy (CLSM) is an invaluable technique available to modern biology for examining single living cells in real-time, using the growing arsenal of vital fluorescent probes [8–10].

The invasiveness of only a few pathogens has been studied intensively, and little is known about this process in M. penetrans. The present study used the CLSM approach to investigate the penetration of M. penetrans into HeLa cells, the subsequent activation of protein kinase C (PKC) and the induction of vacuole formation in the host cells.

**Materials and methods**

*Mycoplasma cultivation*

M. penetrans GTU 54-64-1 was obtained from Dr S-C. Lo (US Armed Forces Institute of Pathology, Washington, DC, USA). Cells were grown for 18–20 h at 37°C in modified Chanock medium [11] supplemented with heat-inactivated horse serum (Biological Industries, Beit Haemek, Israel) 10%. The cells were harvested at the mid-exponential phase of growth (A640 = 0.100.12; pH 7.0) by centrifugation for 20 min at 12,000 , washed once and resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM, Biological Industries). The number of viable cells was determined by plating [1] and expressed as cfu/ml.
Invasion assay

Invasion was assayed by immunofluorescence staining, with a polyclonal rabbit anti-M. penetrans antiserum, kindly provided by J.G. Tully (NIAID, Bethesda, MD, USA). HeLa cells were infected with M. penetrans at a multiplicity of infection (MOI) of 10 as described previously [3]. Washed monolayers of the infected HeLa cells on coverslips were fixed at room temperature for 10 min with freshly prepared formaldehyde 4% in phosphate-buffered saline (PBS, pH 7.4) containing 2 mM MgCl₂. The cells were then washed twice with PBS and incubated for 5 min in 50 mM NH₄Cl to quench free aldehyde groups. After two additional washings with PBS, the cells were permeabilised by incubation for 3 min with Triton X-100 0.2% in PBS-BSA buffer. In some experiments, cells were fixed and permeabilised with absolute methanol at −20°C for 3 min. The coverslips were then overlaid for 20 min at room temperature with normal goat serum 2% and after the excess goat serum was removed, the cells were incubated for 60 min at room temperature with rabbit polyclonal anti-M. penetrans antiserum diluted 1 in 120 in PBS-BSA buffer. Non-bound antibody was removed by dipping the coverslips four times in PBS and the cells were then incubated for 60 min at room temperature with goat anti-rabbit FITC-conjugated IgG serum (Sigma), diluted 1 in 150 in PBS-BSA buffer. The coverslips were rinsed with PBS and mounted in a solution containing glycerol 90%, 1,4-diazabicyclo[2,2,2]-octane 3% as an anti-fading agent and sodium azide 0.1%. The specificity of immunostaining was evaluated by omitting the anti-mycoplasma antibodies or by using non-specific antibodies (non-immune rabbit serum, or anti-M. fermentans antiserum).

Immunofluorescence localisation of protein kinase C (PKC)

HeLa cells treated with phorbol 12-myristate 13-acetate (TPA) or M. penetrans cell fractions were fixed and permeabilised for 3 min at −20°C in absolute methanol and then washed once with PBS. The cells were then incubated for 20 min with normal goat serum 2% in PBS followed by incubation for 1 h at room temperature with anti α, δ and ε PKC-specific antibodies diluted 1 in 100 in PBS containing Triton X-100 0.1%. Non-bound antibody was removed and the cells were treated with the second antibody as described above.

Fluorescence analysis of vacuoles

Two fluorescent stains were used to characterise the vacuolation of HeLa cytoplasm upon internalisation of M. penetrans. For vital staining, live infected HeLa cells were incubated in DMEM containing 5 μM defatted BSA and 5 μM N-[7-(4-nitrobenzol-2-oxa-1,3-diazole)aminocaproyl sphingosine (C₆-NBD-ceramide, Molecular Probes, Eugene, OR, USA) for 20 min at 4°C and then for 30 min at 37°C [12]. For staining permeabilised cells, infected HeLa cells were fixed with absolute methanol (3 min, −20°C), washed once with PBS and incubated for 30 min at 37°C with 3,3′-diododecyloxycarbocyanine perchlorate (DiOC₁₈, Molecular Probes) 10 μg/ml. The stained cells were quickly rinsed in PBS and analysed by confocal microscopy.

Immunoblotting analysis

Proteins of the soluble and particulate fractions of normal HeLa cells, M. penetrans-stimulated and TPA-stimulated HeLa cells were subjected to SDS-PAGE as described by Laemmli [13]. The proteins were transferred to nitrocellulose membranes (BA85, Schleicher and Schuele) with a Hoefer electrophoretic transfer unit according to the manufacturer’s recommendations. To analyse PKC, monoclonal anti-α PKC and goat anti-rabbit IgG peroxidase-conjugated antibodies (Jackson Immuno Research) were used with ECL™ Western blotting detection reagents (Amersham International) according to the manufacturer’s instructions.

Analytical methods

Total protein was determined according to Bradford [14]. To assess the number and viability of HeLa cells, the cells were detached from the wells by EDTA-trypsin treatment, suspended in trypan blue 0.15% solution and analysed microscopically. The HeLa cells were disrupted by brief (1−2 s) ultrasonic treatment in lysis buffer containing 10 mM Tris-HCl, 1 mM EDTA, phenylmethylsulphonyl fluoride (PMSF) 0.1 mg/ml and leupeptine 10 μg/ml (pH 7.5), followed by differential centrifugation to separate the soluble and the particulate cell fractions [15]. Organic peroxides were determined fluorimetrically as described previously [16]. Immunofluorescent samples were analysed with a Sarastro Phoibos 1000 laser scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA, USA) equipped with an argon ion laser tuned at 488 nm and attached to a Universal Zeiss epifluorescence microscope with an oil-immersed Plan Apo 63 × 1.4 N.A. objective lens. Fluorescence was collected at above 515 nm for FITC (with a long-pass barrier filter 515 EFLP). The samples were scanned optically at 0.6 μm increments through the Z-axis and sequential images were collected with a Personal Iris computer (Silicon Graphics).

Results

Confocal analysis of mycoplasma–HeLa cell interaction

The invasion of HeLa cells by M. penetrans was investigated by immunofluorescence staining followed by analysis with CLSM. As can be seen in Fig. 1, when M. penetrans-infected HeLa cells were fixed with formaldehyde and incubated with anti-M. penetrans...
PKC ACTIVATION AND VACUOLATION OF HeLa CELLS BY M. PENETRANS

Fig. 1. Confocal micrographs demonstrating internalisation of M. penetrans by HeLa cells. Following infection with M. penetrans (MOI = 10) for 0, 2 and 4 h, HeLa cells were washed, fixed and immunostained with anti-M. penetrans antibodies as described in Materials and methods. Row A, cells fixed with formaldehyde; B, cells fixed with formaldehyde and permeabilised with Triton X-100; C, cells fixed and permeabilised with methanol. Each picture is a single image made through the mid-X-Y plane of a single HeLa cell.

antibodies followed by a second FITC-conjugated antibody, numerous foci of fluorescence were observed on the cell surface, corresponding to extracellular bound mycoplasmas. An intracellular fluorescent signal was not detected, indicating that the antibodies did not penetrate the host cells. Treatment with Triton X-100 or methanol renders the cells permeable, allowing both extracellular and intracellular mycoplasmas to be stained immunofluorescently (Fig. 1, rows B and C).

Internalisation was not detected in control HeLa cells treated with heat-inactivated (70°C, 10 min) or formaldehyde fixed (4%, 3 min) M. penetrans cells (data not shown).

To obtain three-dimensional information on the cellular location of the fluorescence, a series of optical sections was made through infected HeLa cells. The results clearly demonstrated surface binding
in formaldehyde-fixed HeLa cells, whereas both surface binding and internalisation were observed in fixed cells permeabilised by Triton X-100 (data not shown). The cytoplasmic pool of ingested mycoplasmas was organised in bright fluorescent aggregates that were randomly distributed, tending to concentrate near the plasma membrane.

Table 1 shows the quantitative analysis of the fluorescence intensity of the mycoplasmas–HeLa cell interaction, where formaldehyde and methanol fixations were used to observe extracellularly adhering, and both adhering and internalised mycoplasmas, respectively. The differences between HeLa cells fixed with formaldehyde and those fixed and permeabilised with methanol represents intracellularly located M. penetrans. The amounts of both adhering and intracellularly located mycoplasmas increase during the first 4 h of infection. Adherence and internalisation of M. penetrans by HeLa cells were temperature dependent. Thus, at 4°C, fluorescence was predominantly associated with the HeLa cell surface (data not shown), whereas at 37°C, the fluorescence level associated with the HeLa cells was 10-fold higher than that at 4°C, with most of the fluorescence intensity (c. 75%) detected intracellularly. Taxol, vinblastin and cytochalasin D did not affect the adherence of M. penetrans to HeLa cells, as determined by measuring fluorescence intensity at 4°C (data not shown).

The involvement of the microtubule network in the M. penetrans invasion process was investigated with the microtubule-stabilising agent taxol, which prevents microtubule re-arrangement [17]. Confocal microscopy revealed that treatment of HeLa cells with taxol caused moderate cellular retraction and flattening of the cells, indicative of microtubule network disruption (data not shown) and a drastic reduction in the number of ingested M. penetrans (Table 1). Similar results were obtained after pretreatment of HeLa cells with vinblastine (210 µg/ml, data not shown). The number of ingested M. penetrans was reduced to a lesser extent by treating HeLa cells with cytochalasin D.

Table 1. Binding to and invasion of HeLa cells by M. penetrans

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Cell fixation</th>
<th>Fluorescence (arb. units after incubation for h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Formaldehyde</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>3.7</td>
</tr>
<tr>
<td>Taxol</td>
<td>Formaldehyde</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>2.9</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>Formaldehyde</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>3.2</td>
</tr>
</tbody>
</table>

PKC activation

Activation of PKC is thought to involve redistribution of the enzyme from a cytosolic location in resting cells to a membrane-associated site during stimulation [18]. Therefore, this study examined PKC localisation by an immunofluorescence technique, as well as by immunoblotting proteins of the soluble and particulate fractions of HeLa cells. As multiple discrete subspecies of PKC exhibit different enzymological properties, tissue expression and subcellular localisation after cell stimulation, the cells were stained with α, δ and ζ specific anti-PKC antibodies. In untreated HeLa cells αPKC was located primarily in the cytosol (Fig. 2A), identical results were obtained with δ and ζ PKC (data not shown). After exposure to viable M. penetrans cells for 20 min, translocation of the PKC from the cytosol to the plasma membrane was obtained (Fig. 2c). Translocation was also detected in TPA-stimulated HeLa cells (Fig. 2B) and in HeLa cells stimulated with M. penetrans pretreated with trypsin or proteinase K (25 µg/ml of cell protein) for 30 min at 37°C (data not shown). Partial translocation was observed after stimulation with isolated membrane preparations (data not shown). However, translocation was not detected in HeLa cells treated with M. penetrans in the presence of anti-M. penetrans antiserum (Fig. 2D) or with heat-inactivated (70°C, 10 min) or formaldehyde (4%, 3 min)-treated M. penetrans cells (data not shown). Immunoblots showing the distribution of PKC in the soluble (cytosolic) and particulate (plasma membrane) fractions of HeLa cells treated with M. penetrans or TPA are shown in Fig. 3. Upon stimulation with TPA the 80-kDa immunoreactive protein (i.e., PKC) in the soluble fraction was translocated to the particulate fraction (Fig. 3B). However, only a partial translocation was detected when HeLa cells were treated with M. penetrans cells (Fig. 3C).

Vacuolation of infected HeLa cells

HeLa cells displayed no detectable morphological alterations at the end of a 2-h infection period, but after 4 h, infected HeLa cells developed progressive cytoplasmic vacuolation (Fig. 4). The vacuoles appeared to be empty, differing from the described membrane-bound vesicles containing clusters of bacteria [19]. The amount and size of the vacuoles depended on the duration of infection (Table 2). Table 2 also shows that vacuolation was not detected in HeLa cell cultures treated with formaldehyde-fixed M. penetrans, mycoplasmal growth medium (10100 µl/ml growth medium) obtained after harvesting M. penetrans or a soluble extract (protein 10100 µg/ml) obtained from M. penetrans cells disrupted by ultrasonic treatment [11]. Vacuolation was prevented by anti-M. penetrans antiserum (data not shown). Nonetheless, a low extent of vacuolation was seen after treating HeLa cells with M. penetrans membranes (protein 10 µg/ml). Prolonged incubation of HeLa cells with M. penetrans (8 h) was also characterised by
progressive swelling of the HeLa cells and lack of cell proliferation (data not shown). Non-infected HeLa cells did not display any detectable alterations during the same incubation periods.

It was shown previously that degenerative processes in eukaryotic cells are accompanied by elevated levels of free radicals and the accumulation of lipid peroxides [20]. Therefore, the present study analysed HeLa cells incubated for various periods of time with \textit{M. penetrans} for organic peroxide concentrations. As shown in Fig. 5, HeLa cells infected with \textit{M. penetrans} accumulated organic peroxides in a time-dependent manner. Very low levels of organic peroxides were detected in uninfected HeLa cells. To test the possible role of organic peroxides in the vacuolation process, HeLa cells infected by \textit{M. penetrans} were grown with or without the lipid soluble antioxidant \textit{\textalpha;}-tocopherol and the extent of vacuolation was determined (Table 3). \textit{\textalpha;}-Tocopherol partially prevented peroxide accumulation in the infected HeLa cells, resulting in reduced vacuolation. The addition of \textit{\textalpha;}-tocopherol to the DMEM medium did not affect HeLa cell viability, as determined by trypan blue staining, or the degree of \textit{M. penetrans} ingestion by HeLa cells, as assessed by plating [3].

### Discussion

In studying bacterial invasion, it is essential to differentiate between micro-organisms adhering to a...
Fig. 3. Immunoblots showing PKC in the soluble and particulate fractions of HeLa cells treated with TPA and M. penetrans. HeLa cells were treated for 20 min with TPA (10 ng/ml) or M. penetrans (MOI = 10). The cells were then disrupted and the soluble and particulate fractions were subjected to SDS-PAGE (20 μg protein/lane), blotted onto a nitrocellulose membrane and incubated with anti-PKC (1 in 25 000 dilution) followed by a secondary anti-rabbit IgG peroxidase-conjugated antibody (1 in 25 000 dilution). The immunoreactive proteins were visualised by ECL™ detection reagent as described in Materials and methods. A, untreated control cells; B, TPA-treated cells; C, M. penetrans-treated cells; s, soluble fraction; p, particulate fraction.

Fig. 4. Vacuolation of HeLa cells infected with M. penetrans. HeLa cells were incubated at 37°C with M. penetrans (MOI = 10) and stained with DiOC18 as described in Materials and methods. A, uninfected HeLa cells; B, 24 h after infection.

Table 2. Vacuolation of HeLa cells infected with M. penetrans

<table>
<thead>
<tr>
<th>HeLa cells incubated with</th>
<th>Percentage of vacuolated HeLa cells after 4 h</th>
<th>Percentage of vacuolated HeLa cells after 12 h</th>
<th>Percentage of vacuolated HeLa cells after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact M. penetrans</td>
<td>3</td>
<td>16</td>
<td>47</td>
</tr>
<tr>
<td>M. penetrans pretreated with 4% formaldehyde</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>M. penetrans membrane fraction</td>
<td>4</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>M. penetrans soluble fraction</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>M. penetrans growth medium</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

HeLa cell cultures (10⁶ cells) were incubated with intact M. penetrans (MOI = 10), M. penetrans growth medium (100 μl), fixed M. penetrans or M. penetrans cell fractions (10 μg protein/ml) for various periods of time. They were then fixed and stained with DiOC18 and the extent of vacuolation was determined as described in Materials and methods.

A previous study demonstrated that invasion of HeLa cells is totally dependent on the capacity of the cells to assemble actin microfilaments, as treatment with cytochalasin D inhibited invasion by M. penetrans cells [3]. This observation is supported by the results of the present confocal studies. Furthermore, as taxol, a drug known to disorganise microtubules and vinblastine, which disrupts microtubules, virtually abolished the penetration of M. penetrans, it seems that alterations in polymerisation dynamics and stability of both microfilaments and microtubules have a dramatic effect on the invasion process. Drugs that disrupt microtubules may inhibit contractile intracellular processes such as the invagination and transport of membrane-bound bacteria from the plasma membrane into the cell, either by preventing their movement along the microtubules or by inhibiting the necessary actin-mediated contractile forces [23, 24]. Consistent
with previous observations [8] adherence of *M. penetrans* to HeLa cells was not inhibited by cytochalasin D, vinblastine or taxol.

Very little is known about the molecular events associated with the invasion process. A recent study showed that invasion of HeLa cells by *M. penetrans* is associated with tyrosine-phosphorylation of a 145-kDa protein [3]. As *M. penetrans* possesses a potent phospholipase C [8, 25], it is likely that the interaction of *M. penetrans* with HeLa cells results in the activation of PKC due to the generation of diacylglycerol [26, 27]. PKC has attracted much attention because of its pivotal role in signal transduction [8, 29]. Activation of the enzyme by various agonists results in its translocation from the cytosol to the particulate fraction, including the plasma membrane, cytoskeletal elements and nucleus [18, 30]. Therefore, measurement of the extent of PKC translocation in HeLa cells was used to monitor the enzyme’s activation by *M. penetrans*. The present confocal microscopy study clearly demonstrated translocation of PKC from the cytosol to the plasma membrane as early as 20 min after the interaction between *M. penetrans* and the host cells. When activation-induced translocation of PKC was examined in independent HeLa cell experiments with cell fractionation in the presence of a Ca\(^{2+}\) chelator and Western blot analysis, translocation following induction by *M. penetrans* cells was less pronounced than after induction with TPA. The difficulty in detecting pronounced *M. penetrans*-induced translocation of PKC by immunoblotting may reflect differences between the methods used in the two studies. An intriguing explanation could be that the movement of some activated PKC from one cell compartment to the other is not associated with tight binding of the kinase to the particulate fraction. It seems that the PKC of HeLa cells activated upon *M. penetrans* invasion is a reversibly bound enzyme form which associates with the membrane by a delicate equilibrium [28]. These data highlight the importance of immunolocalisation as a fine means of determining PKC activation-induced translocation. *M. penetrans* has been shown to be cytopathic [4] and to actively invade cells in culture [3, 9]. However, the pathogenic properties of this organism in vivo are still unclear. The activation of PKC described in the present study may be significant within the context of the interaction of *M. penetrans* and the host target cell in vivo, as PKC effectively mediates a wide range of biological activities in cell proliferation and cell differentiation of various cells [27].

Bacterial attachment to eukaryotic cells, even without subsequent entry, may lead to a pronounced cytotoxic effect [31]. In the case of *M. penetrans*, cytopathological effects were observed 2–5 days after infection [19]. In the present study vacuolation of HeLa cells infected by *M. penetrans* was detected as early as 4 h after infection. As vacuolation was not obtained with *M. penetrans* cell fractions or with the growth medium derived after harvesting *M. penetrans* cells, it is unlikely that a necrotising cytotoxin was involved in the generation of the cellular lesions, as suggested in other systems [32, 33]. A possible mechanism that might lead to vacuolation is the accumulation of organic peroxides upon invasion of HeLa cells by *M. penetrans*. It has been shown previously that degenerative processes in eukaryotic cells are accompanied by elevated levels of free radicals and an accumulation of lipid peroxides [20]. The observation that when HeLa cells were grown with an antioxidant the level of accumulated organic peroxides was very low and vacuolation was almost completely abolished, suggests that the primary reason for vacuolation may indeed be the accumulation of such compounds.
This study was supported by grant no. 3253 from the Israel Ministry of Health.

References