MICROBIAL PATHOGENICITY

Protein kinase C activation and vacuolation in HeLa cells invaded by *Mycoplasma penetrans*

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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 immunochemistry 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 ($A_{640} = 0.100.12$; 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]-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 ζ 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 μ M defatted BSA and 5 μ M 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'-dioctadecyloxycarbocyanine perchlorate (DiOC₁₈, Molecular Probes) 10 μ g/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 ECLTM 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 μ g/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 $\times$ 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 μ m 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*

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*

Cell treatment	Cell fixation	Fluorescence (arb. units after incubation for (h))		
		0	2	4
None	Formaldehyde	3.1	10.5	12.1
	Methanol	3.7	40.8	51.0
Taxol	Formaldehyde	3.1	3.8	4.6
	Methanol	2.9	6.3	3.7
Cytochalasin D	Formaldehyde	2.6	9.2	8.6
	Methanol	3.2	17.5	19.7

HeLa cells treated with taxol or cytochalasin D (5 µg/ml of each) were incubated for up to 4 h with *M. penetrans* (MOI = 10) at 37°C. The cells were then fixed with formaldehyde or permeabilised and fixed with methanol, immunostained and fluorescence was determined as described in *Materials and methods*.

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 α PKC 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/mg 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*, mycoplasma 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

Fig. 2. Immunofluorescence localisation of α PKC in HeLa cells. HeLa cells were treated for 20 min with TPA or *M. penetrans* and then fixed and stained as described in *Materials and methods*. **A**, non-stimulated cells; **B**, TPA-stimulated cells (10 ng/ml); **C**, *M. penetrans*-stimulated cells (MOI = 10); **D**, cells treated with *M. penetrans* in the presence of anti-*M. penetrans* antiserum. Arrows, staining at the cell periphery.

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 *M. penetrans* for organic peroxide concentrations. As shown in Fig. 5, HeLa cells infected with *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 *M. penetrans* were grown with or without the lipid soluble antioxidant α -tocopherol and the extent of vacuolation was determined (Table 3). α -Tocopherol partially prevented peroxide accumulation in the infected HeLa cells, resulting in reduced vacuolation. The addition of α -tocopherol to the DMEM medium did not affect HeLa cell viability, as determined by trypan blue staining, or the degree of *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 on to 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 ECLTM 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.

host cell and those that have been internalised. The immunofluorescence quantitative technique is used to detect the internalised invaders. This rather simple and reliable method is based on the differential fluorescent staining of internalised bacteria and those that remain on the cell surface [21, 22]. In the present study, immunofluorescence combined with CLSM was applied to demonstrate that *M. penetrans* is capable of penetrating HeLa cells. By means of this non-destructive, high-resolution method infected HeLa cells were optically sectioned, following fixation and immunofluorescent labelling, to localise mycoplasmas within the host cell. Single-cell imaging of infected HeLa cells revealed that the invasion was a time- and temperature-dependent process. Penetration of the HeLa cells was observed as early as 20 min after infection, whereas invasion of cultured Hep-2 cells by *M. penetrans* has been shown to begin 2 h after infection [9].

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

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*

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

HeLa cell cultures (10^6 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*.

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

Fig. 5. Organic peroxides accumulated in HeLa cells upon infection with *M. penetrans*. HeLa cells were incubated for up to 24 h at 37°C with *M. penetrans* (MOI = 10). Organic peroxides were determined as described in *Materials and methods*. Open symbols, non-infected cells; closed symbols, infected cells.

Table 3. The effect of an antioxidant on organic peroxide level and vacuolation of HeLa cells infected with *M. penetrans*

Preparation	Organic peroxides (arb. units/mg protein)	Vacuolation (percent of cells vacuolated)
Uninfected cells	92	2
Infected cells	224	39
α -Tocopherol-treated infected cells	114	7

HeLa cells were incubated with *M. penetrans* (MOI = 10) for 24 h as described in *Materials and methods*. α -Tocopherol (final concentration 2 μ M) was added to HeLa growth medium simultaneously with *M. penetrans*. Organic peroxide and vacuolation were determined as described in *Materials and methods*.

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 [28, 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 cytopathic 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.

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References

1. Razin S, Jacobs E. Mycoplasma adhesion. *J Gen Microbiol* 1992; **138**: 407–422.
2. Taylor-Robinson D, Davies HA, Sarathchandra P, Furr PM. Intracellular location of mycoplasmas in cultured cells demonstrated by immunocytochemistry and electron microscopy. *Int J Exp Pathol* 1991; **72**: 705–714.
3. Andreev J, Borovsky Z, Rosenshine I, Rottem S. Invasion of HeLa cells by *Mycoplasma penetrans* and the induction of tyrosine phosphorylation of a 145-kDa host cell protein. *FEMS Microbiol Lett* 1995; **132**: 189–194.
4. Lo SC. Mycoplasma and AIDS. In: Maniloff J, McElhaney RN, Finch LR, Baseman JB (eds) *Mycoplasmas: molecular biology and pathogenesis*. Washington, DC, ASM Press. 1992: 525–548.
5. Falkow S, Isberg RR, Portnoy DA. The interaction of bacteria with mammalian cells. *Annu Rev Cell Biol* 1992; **8**: 333–363.
6. Bliska JB, Galán JE, Falkow S. Signal transduction in the mammalian cell during bacterial attachment and entry. *Cell* 1993; **73**: 903–920.
7. Tang P, Foubister V, Pucciarelli MG, Finlay BB. Methods to study bacterial invasion. *J Microbiol Methods* 1993; **18**: 227–240.
8. Baseman JB, Lange M, Criscimagna NL, Giron JA, Thomas CA. Interplay between mycoplasmas and host target cells. *Microb Pathog* 1995; **19**: 105–116.
9. Giron JA, Lange M, Baseman JB. Adherence, fibronectin binding, and induction of cytoskeleton reorganization in cultural human cells by *Mycoplasma penetrans*. *Infect Immun* 1996; **64**: 197–208.
10. Terasaki M, Dailey ME. Confocal microscopy of living cells. In: Pawley JB (ed) *Handbook of biological confocal microscopy*, 2nd edn. New York and London, Plenum Press. 1995: 327–346.
11. Salman M, Rottem S. The cell membrane of *Mycoplasma penetrans*: lipid composition and phospholipase A₁ activity. *Biochim Biophys Acta* 1995; **1235**: 369–377.
12. Martin OC, Comly ME, Blanchette-Mackie EJ, Pentchev PG, Pagano RE. Cholesterol deprivation affects the fluorescence properties of a ceramide analog at the Golgi apparatus of living cells. *Proc Natl Acad Sci USA* 1993; **90**: 2661–2665.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
14. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
15. Rosenshine I, Ruschowski S, Stein M, Reinscheid DJ, Mills SD, Finlay BB. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. *EMBO J* 1996; **15**: 2613–2624.
16. Salman M, Shirazi I, Tarshis M, Rottem S. Fusion of *Spiroplasma floricola* cells with small unilamellar vesicles is dependent on the age of the culture. *J Bacteriol* 1993; **175**: 6652–6658.
17. Rikihisa Y, Zhang Y, Park J. Inhibition of infection of macrophages with *Ehrlichia risticii* by cytochalasins, monodansylcadaverine, and taxol. *Infect Immun* 1994; **62**: 5126–5132.
18. Disatnik M-H, Buraggi G, Mochly-Rosen D. Localization of protein kinase C isozymes in cardiac myocytes. *Exp Cell Res* 1994; **210**: 287–297.
19. Lo SC, Hayes MM, Kotani H et al. Adhesion onto and invasion into mammalian cells by *Mycoplasma penetrans*: a newly isolated mycoplasma from patients with AIDS. *Mod Pathol* 1993; **6**: 276–280.
20. Boobis AR, Fawthrop DJ, Davies DS. Mechanisms of cell death. *Trends Pharmacol Sci* 1989; **10**: 275–280.
21. Heesemann J, Laufs R. Double immunofluorescence microscopic technique for accurate differentiation of extracellularly and intracellularly located bacteria in cell culture. *J Clin Microbiol* 1985; **22**: 168–175.
22. Hondalus MK, Mosser DM. Survival and replication of *Rhodococcus equi* in macrophages. *Infect Immun* 1994; **62**: 4167–4175.
23. Finlay BB, Ruschowski S, Dedhar S. Cytoskeletal rearrangements accompanying *Salmonella* entry into epithelial cells. *J Cell Sci* 1991; **99**: 283–296.
24. Guzman CA, Rohde M, Timmis KN. Mechanisms involved in uptake of *Bordetella bronchiseptica* by mouse dendritic cells. *Infect Immun* 1994; **62**: 5538–5544.
25. Shibata K-I, Sasaki T, Watanabe T. AIDS-associated mycoplasmas possess phospholipases C in the membrane. *Infect Immun* 1995; **63**: 4174–4177.
26. Meyers DJ, Berk RS. Characterization of phospholipase C from *Pseudomonas aeruginosa* as a potent inflammatory agent. *Infect Immun* 1990; **58**: 659–666.
27. Parkinson JS. Signal transduction schemes of bacteria. *Cell* 1993; **73**: 857–871.
28. Azzi A, Boscoboinik D, Hensey C. The protein kinase C family. *Eur J Biochem* 1992; **208**: 547–557.
29. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 1992; **258**: 607–614.
30. Mochly-Rosen D. Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science* 1995; **268**: 247–251.
31. Kelly RB. Microtubules, membrane traffic, and cell organization. *Cell* 1990; **61**: 5–7.
32. Fiorentini C, Donelli P, Matarrese A, Fabbri A, Paradisi S, Boquet P. *Escherichia coli* cytotoxic necrotizing factor 1: evidence for induction of actin assembly by constitutive activation of the p21 Rho GTPase. *Infect Immun* 1995; **63**: 3936–3944.
33. Ghiara P, Marchetti M, Blaser MJ, et al. Role of the *Helicobacter pylori* virulence factors vacuolating cytotoxin, cagA, and urease in a mouse model of disease. *Infect Immun* 1995; **63**: 4154–4160.