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Fusion of *Mycoplasma fermentans* strain incognitus with T-lymphocytes

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The ability of *Mycoplasma fermentans* (strain incognitus) to fuse with cultured lymphocytes was investigated and the fusion process was characterized. Fusion was measured using an assay to determine lipid mixing based on the dequenching of the fluorescent probe, octadecylrhodamine (R18), that was incorporated into the mycoplasma cells. Fusion of *M. fermentans* was detected with both $CD4^+$ (Molt 3) and $CD4^-$ (12-E1) cells. The amount of fusion induced was relatively low and ranged from 5–10% with either cell culture. When primary peripheral blood lymphocytes were used the fusion yield was somewhat higher, reaching 12% of the cell population. Similar findings were obtained with fluorescent microscopy analysis suggesting that a predetermined, but unidentified subpopulation of cultured lymphocytes, were being fused. The rate of fusion was temperature dependent. Following a short lag period fusion at 37°C was virtually completed in 60 min. The lymphocytes remained intact throughout the fusion process, as determined by the Trypan blue staining procedure. Fusion was almost completely inhibited by anti-*M. fermentans* antisera and by pretreatment of *M. fermentans* cells with proteolytic enzymes, suggesting that a surface-exposed proteinaceous component is involved in the fusion process.

Mycoplasma fermentans; Fusion; Lymphocyte; AIDS

1. INTRODUCTION

Lo and co-workers [1] have isolated a virus-like infectious agent (VLIA) from a patient with AIDS. This agent was later identified as a unique strain of *Mycoplasma fermentans* and was designated the incognitus strain [2]. The observations were later confirmed [3] that mycoplasmas may play a key role in one of the early stages of the lentivirus life cycle, namely absorption, penetration, provirus synthesis, or early repression. It was, therefore, suggested that the modification of the biological properties of HIV-1 by co-infection with mycoplasma may be involved in the pathogenesis of acquired immunodeficiency syndrome (AIDS) [4]. In fact introduction of mycoplasmas to T-cell line CEM cultures significantly reduced CD4 expression in consequent inhibition of gp120 binding and HIV infection of CEM cells [5], and treatment of HIV-1-infected T-cells with tetracyclines caused a decrease in HIV-1 replication as measured by reverse transcriptase activity [6].

Mycoplasmas have been previously shown to affect multiple aspects of cell function and activities, including the ability to stimulate or inhibit viral infections in infected cell cultures, as well as in animals [7,8]. Several mechanisms were postulated for the effects on viral growth, including (i) depletion of arginine from the medium by arginine-utilizing mycoplasmas decreasing viral yields, (ii) inhibition of interferon induction and activity increasing yields of interferon-sensitive viruses, (iii) removal of essential lipid components from the infected eukayrotic cells by the avidly attached pathogenic mycoplasma and (iv) induction of cytokine and lymphokine release from eukaryotic cells stimulated by the attachment of mycoplasmas to the cell surface. Such a release might produce a hypoactive state of the host tissues making them more susceptible to viral infection [8]. In the present study we have shown that the AIDS-associated strain incognitus of *M. fermentans* is capable of fusing with the T-cell line cells, as well as with peripheral blood lymphocytes. We suggest, therefore, that the possible role of *M. fermentans* strain incognitus in the pathogenesis of AIDS may be attributed, at least in part, to the delivery of mycoplasma components into the lymphocytes upon mycoplasma–lymphocyte fusion.

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2. MATERIALS AND METHODS

2.1. *Organisms, cell cultures and growth conditions*

Mycoplasma fermentans (strain incognitus) was grown in Channock medium supplemented with 10% horse serum [9]. The cultures were grown for 48–72 h at 37°C. The organisms were collected by centrifugation at 12,000 × *g* for 20 min, washed twice and resuspended in cold phosphate-buffered saline (PBS, pH 7.5) to a concentration of 1 mg cell protein/ml. The human cell line $CD4^+$ Molt 3 cells were obtained from the American Type Culture Collection (Rockville, MD), and $CD4^-$ 12-E1 cells were provided by Dr. H. Goldring (FDA, Bethesda, MD). The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

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2.2. *Fusion analyses*

Fusion of *M. fermentans* with the Molt 3 and 12-E1 cells was monitored by the octadecylrhodamine B chloride (R18, Molecular Probes, Eugene, OR) assay described before [10]. For the labeling of *M. fermentans* with R18, 10 μl of an ethanolic solution of R18 (1 mg/ml) was added to 1 ml of a mycoplasma cell suspension containing 1 mg cell protein/ml, and incubated at room temperature for 15 min in the dark. The cells were then harvested and washed twice by centrifugation in an Eppendorf centrifuge for 3 min. The R18-labelled *M. fermentans* cell suspension (10–20 μl) was rapidly mixed with 1 ml of T-cell line cells pre-cooled to 4°C (10^6 cells/ml). The temperature of the mixture was maintained at 4°C for 30 min and the cells were then sedimented by centrifugation, washed twice and resuspended in 1 ml of cold PBS. The cell suspension was then transferred to 37°C and the intensity of fluorescence dequenching was measured with an excitation and emission wave length of 560 and 590 nm, respectively. The dequenching degree obtained in the presence of 0.1% Triton X-100 was taken to present 100% dequenching, i.e. infinite dilution of the probe. Phase contrast and epifluorescence microscopy observations were performed as described before [11].

2.3. *Analytical methods*

Protein was determined by the method of Bradford [12] utilizing the Bio-Rad reagent with bovine serum albumin as the standard. Treatment of *M. fermentans* with proteolytic enzymes was performed by incubating 1 mg of *M. fermentans* cell protein with trypsin (50 μg/ml) or proteinase K (50 μg/ml) for 30 min at 37°C. NADH dehydrogenase activity was measured by following the decrease in absorbance at 340 nm of a NADH solution incubated with sodium deoxycholate-treated *M. fermentans* cell lysate [13].

3. RESULTS AND DISCUSSION

When R18-labeled *M. fermentans* cells were mixed in the cold with target Molt 3 cells no labeled target cells were detected by fluorescent video microscopy. However, 20 min after the temperature was raised to 37°C, 10–25% labeled cells were detected (Fig. 1). Fig. 2 shows the kinetics of membrane fusion of *M. fermentans* cells and Molt 3 cells. The percent of dequenching of R18 (fusion yield) was measured at different periods of time following incubation of the mycoplasmas pre-bound to the target Molt 3 cells at 37°C. After a brief lag period (1–2 min) fusion proceeded exponentially for about 30 min. Maximal fusion yields were obtained after about 40–60 min incubation at 37°C. Polyethylene glycol (PEG 8000), previously shown to be necessary for the fusion of *M. capricolum* [10] with lipid vesicles, has an effect on the lag period. In the presence of 4% PEG the lag period was not affected, whereas with 10% PEG no lag period was observed (data not shown). It has been previously proposed that PEG alters the physical state of either the bulk water adjacent to the cell surface or the water of hydration of the phospholipid polar head groups in the cell membrane [14]. 12-E1 cells lacking CD4 fused at the same rate and to the same extent as the $CD4^+$ Molt 3 cells (data not shown), suggesting that CD4 is not required on the target membrane for *M. fermentans*-mediated membrane fusion. Very little fluorescence dequenching (≤1%) was observed when T-lymphocytes were incubated with isolated membranes of *M. fermentans* or with intact *M. fermentans* cells pretreated with 0.1% glutaraldehyde. Furthermore, the addition of the H^+ ionophore, carbonyl cyanide *m*-chlorophenylhydrazone (5 μM), to intact *M. fermentans* cells inhibited the extent of fluorescence observed by 60–70%, suggesting that a pH gradient across the mycoplasma cell membrane is required for the fusion process.

M. fermentans cells were rendered non-fusogenic after treatment with proteinase K (50 μg/ml for 30 min at 37°C). Trypsin digestion caused a marked decrease (60–70%) in the degree of fluorescent dequenching. As after either proteinase K or trypsin treatment almost all the NADH dehydrogenase activity was retained within the cells it seems that the proteolytic digestion did not affect cell intactness. The marked effect of proteolytic digestion of *M. fermentans* cells on their capacity to fuse

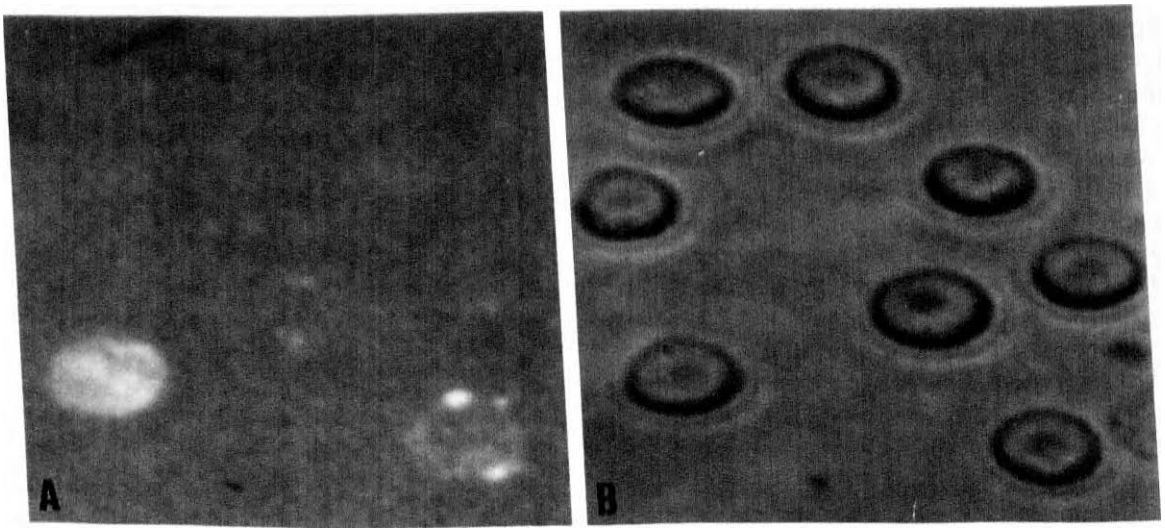

Fig. 1. Fusion of *M. fermentans* (incognitus strain) with Molt 3 cells. Molt 3 cells were mixed with fluorescently labeled mycoplasmas, then washed and incubated for 30 min at 37°C. The panel shows a field of cells under epifluorescence (A) and phase contrast (B).

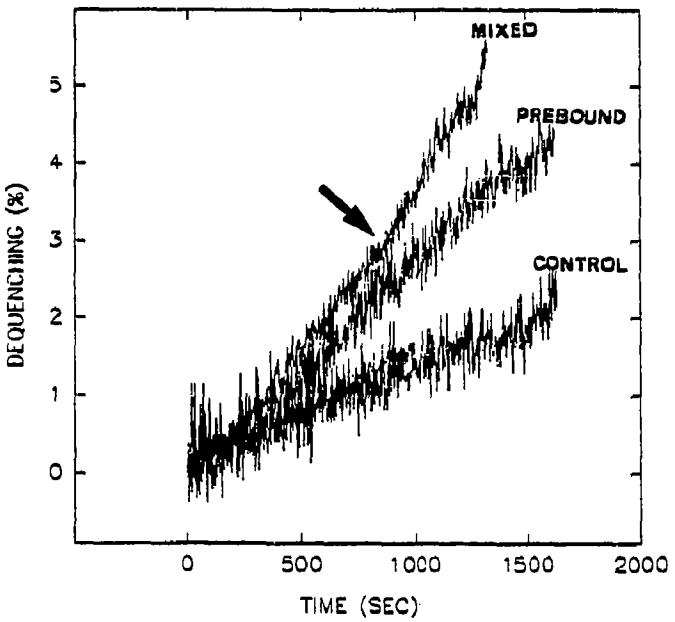

Fig. 2. Kinetics of mycoplasma–Molt 3 fusion. Molt 3 cells (10^5) were mixed with fluorescently labeled mycoplasma (2.4×10^6 CFU/ml). The mixture was either incubated at 37°C and fluorescence intensity measured (MIXED), or pre-incubated at 4°C for 30 min, washed twice, and then transferred to 37°C and fluorescence measured (PREBOUND). In a control experiment, heat-inactivated (56°C, 30 min) mycoplasmas were utilized. At the time indicated by the arrow more Molt 3 cells (10^5) were added to the mixture.

with target Molt 3 cells suggests that *M. fermentans* cells possess proteinase-sensitive receptors on their cell surface responsible for binding and/or for establishing a tighter contact with the cell membrane of Molt 3 cells.

Table I shows that attachment and subsequent fusion of labeled *M. fermentans* was also obtained with peripheral blood lymphocytes (PBL). As the increase in the extent of fluorescence observed by incubating *M. fermentans* with PBL was neither affected by vinblastine (20 μg/ml), known to inhibit microtubule formation, nor by cytochalasin B (20 μg/ml) which interferes with the assembly and disassembly of active filaments (data not shown), it is unlikely that the increased fluorescence was due to phagocytosis of the mycoplasma by activated monocytes present in the PBL. Whereas the binding of *M. fermentans* to either PBL or Molt 3 cells was similar the fusion yield with target PBL was somewhat higher. The relatively low fusion yield was not affected by increasing the amount of R18-labeled *M. fermentans* cells. Nonetheless, the percent of fusion was significantly increased with increasing numbers of target cells. This result would indicate that only a small subpopulation of the T-cells are participating in the fusion event.

Table I

Binding and fusion of *M. fermentans* incognitus strain with PBL and Molt 3 cells at various mycoplasma-to-cell ratios

		10 μl		100 μl	
		PBL	Molt 3	PBL	Molt 3
Binding of *M. fermentans* to cells (a.u.)					
M. fermentans	(10 μl)	0.67	0.82	1.20	1.02
	(100 μl)	3.58	3.96	7.76	7.94
Fusion of *M. fermentans* with cells (%)					
M. fermentans	(10 μl)	1.1	0.8	2.8	1.7
	(100 μl)	1.2	1.1	1.8	1.4

10 or 100 μl cells (PBL or Molt 3) were mixed with 10 or 100 μl of mycoplasmas (2.4×10^7/ml) and incubated at 4°C for 30 min. The mixture was then washed twice, transferred to 37°C and the fluorescence was measured. Lag times of fusion and rates of fusion were approximately the same at all conditions. a.u. = arbitrary units.

Although the role that the AIDS-associated strain incognitus of *Mycoplasma fermentans* plays in the pathogenesis of disease has not been defined our findings may provide meaningful insight regarding the interactions between mycoplasmas and lymphocytes, one of the critical components of the immune system. It is likely that mycoplasma components are being delivered into the lymphocytes upon mycoplasma–lymphocyte fusion, and thereby affecting the normal functions of the lymphocyte. Furthermore, the insertion of mycoplasma membrane components into the membranes of lymphocytes upon fusion could alter receptor recognition sites, as well as affecting the induction and expression of lymphokines, resulting in alterations in the communication between various cellular elements of the immune system. Such effects could influence binding, integration and/or insertion or expression of HIV, decreasing host resistance to infection. Obviously, additional studies are required before the role that *M. fermentans* plays in the pathogeneses of disease can be determined.

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