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In Vitro Influence of Mycoplasma penetrans on Activation of Peripheral T Lymphocytes from Healthy Donors or Human Immunodeficiency Virus-Infected Individuals

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Mycoplasma penetrans is a mycoplasma species newly isolated from the urine of human immunodeficiency virus (HIV)-infected individuals and presents the only case in which an association has been found between antibodies against a mycoplasma and HIV infection. To further explore the effects of M. penetrans on the immune system, we studied the influence of this mycoplasma on peripheral blood mononuclear cells (PBMCs) from healthy donors and HIV-infected individuals. M. penetrans-induced, in addition to blastogenesis of PBMCs, a significant proliferative response associated with the expression of some activation markers such as CD69, HLA-DR, and CD25. This M. penetrans-dependent lymphocyte activation was observed not only in healthy donors but also in HIV-infected persons at different stages of the disease. In addition, our study revealed that both CD4+ and CD8+ T lymphocytes were responsive to M. penetrans. Interestingly, the mitogenic activity of M. penetrans was associated with mycoplasma cells but not with the supernatants of mycoplasma culture. The potent stimulating activity of M. penetrans on T lymphocytes from HIV-infected individuals is of particular interest in view of the supposed contribution of immune activation to HIV replication and disease progression.

One of the hallmarks of human immunodeficiency virus (HIV) infection is a chronic and unbalanced activation of the immune system. This activation affects most of the immune components and can be exemplified by hypergammaglobulinemia (27), increased secretion of cytokines, including tumor necrosis factor alpha and interleukin-6 (IL-6) (13, 16, 26), increased level of serum soluble IL-2 receptor (12), and expression of activation markers such as CD38 and HLA-DR on CD8+ lymphocytes (11, 14, 17) and CD25 on both CD4+ and CD8+ cells (23).

Parallel to cellular activation, T-cell unresponsiveness (anergy) occurs before the numbers of CD4+ cells decline. Functional defects of helper T cells are observed in patient lymphocytes; these are characterized by the impairment of in vitro T-cell-receptor-dependent activation in response to major histocompatibility complex-restricted recall antigen (31) or to anti-CD3 monoclonal antibodies (MAbs) (25).

Various factors are thought to be responsible for the general state of immune activation. HIV itself probably plays a major role in the chronicity of the immune activation (for a review, see reference 6), but it is also recognized that antigenic stimulation from other microorganisms can increase this level of activation (5, 10, 21, 32). It has been shown that Pneumocystis carinii infection is associated with higher viral titers in bronchoalveolar lavage fluids (21) and that there is also an increased viremia in patients following influenza A vaccination (10). There are also indications that mycobacterial infections could hasten the progression of HIV disease by increasing the immune activation (5, 32). The antigenic stimulation found during opportunistic infections is not necessarily associated with clinical manifestations. For example, a recent report indicates that subclinical Mycobacterium avium intracellulare infection is common among HIV-infected patients and can be evidenced by restoration of tuberculin response after zidovudine treatment (24). The possibility that other microorganisms may produce clinically silent infections with activating effects on the immune system and consequently on HIV viremia cannot be excluded.

Mycoplasma penetrans is a newly characterized species of mycoplasma which has been isolated from the urine of patients infected with HIV (19, 20). This mycoplasma exhibits unique morphological characteristics, with a tip-like structure, and has been shown both in vitro and in vivo to have the capacity to invade cells (18). Epidemiological data have shown a high frequency of M. penetrans antibodies in HIV-infected patients (20% in symptom-free subjects and 40% in patients with AIDS versus 0.3% in HIV-negative controls) by enzyme-linked immunosorbent assay with membrane-associated antigens (33, 34). The production of these anti-M. penetrans antibodies was found to be associated with HIV infection in homosexual males (33, 34), and we have obtained similar results with patients recruited in France (8).

Considering the high seroprevalence found for this mycoplasma in persons infected with HIV and because several mycoplasma species have been shown to polyclonally activate human T lymphocytes (1, 2, 4, 28), we studied the effects of M. penetrans on the in vitro activation of peripheral blood mononuclear cells (PBMCs) from healthy donors and HIV-infected individuals.

MATERIALS AND METHODS

Mycoplasma culture. M. penetrans GTU-54-6A1, a gift from J. G. Tully (National Institute of Allergy and Infectious Diseases, Frederick, Md.), was cultured
in PPLO broth containing 10% (vol/vol) heat-inactivated horse serum, 0.25% (wt/vol) glucose, and 0.002% phenol red. This strain was a low-passage derivative (fewer than 10 in vitro passages) of the original isolate. The M. penetrans culture was added as a stimulant for the PBMCs at the concentration indicated in the text. The concentration of mycoplasmas in the cell suspension was tested with an inoculum of $1 \times 10^9$ color-changing units/ml and was found to be stable ($10^8$ color-changing units/ml) over a 5-day culture. This does not exclude the possibility that M. penetrans multiplied and either remained attached to the surface of the PBMCs or penetrated into the cytoplasm of some of these cells. In some experiments, mycoplasma cells and supernatants of the cultures were tested after separation by centrifugation for 20 min at 20,000 $\times g$ at 4°C. The Mycoplasma cells were washed once with RPMI medium and resuspended in the same medium.

**Blood samples.** Blood was obtained from 15 healthy donors (Hôpital Saint Joseph, Paris, France) and 23 HIV-infected individuals, the majority of whom were asymptomatic (16) and two of whom were at stage IV, who were recruited at the Hôpital Militaire Bâine, Paris, France (by R. Roué and T. Debord). PBMCs were isolated from fresh blood by Ficoll-Hypaque centrifugation and cultured in RPMI medium containing 10% (vol/vol) heat-inactivated fetal calf serum, penicillin G (50 U/ml), streptomycin (50 $\mu$g/ml), l-glutamine (1 mM), and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; 10 mM). The bacterial superantigen Staphylococcus enterotoxin type E (SE; Toxin Technology, Inc., Sarasota, Fla.) was used as a positive control at a concentration of 1 $\mu$g/ml for the proliferation assay and the phenotypic analysis.

**Serologic evaluation.** To determine whether HIV-infected subjects had been exposed to M. penetrans, antibodies against this mycoplasma were detected in patient sera by Western blotting (immunoblotting) with membrane antigens that were extracted selectively with the detergent Triton X-114 (33). Among 23 HIV-infected individuals tested for the proliferative response to M. penetrans, 12 corresponding serum samples were available for this serological evaluation.

**Assay for proliferative response.** PBMCs from healthy donors and HIV-infected individuals were tested for the proliferative response against SEE and M. penetrans. A total of $1.5 \times 10^6$ PBMCs were cultured in quadruplicate in 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, N.Y.), in 0.2-ml volumes, in the presence of viable mycoplasmas in cell suspension or mycoplasma cell fractions (cell supernatant or mycoplasmal cells) for various periods of time. Cell proliferation was measured by pulsing lymphocyte cultures with 1 $\mu$Ci of [3H]thymidine per well for the last 16 h of the culture. Data are presented as the average counts per minute incorporated in quadruplicate cultures. To evaluate the specificity of thymidine incorporation, irradiated PBMCs (3,000 rads) were infected with mycoplasmas, cultured as described above, and then pulsed with [3H]thymidine. Data were calculated statistically (mean, standard error, and P value) by the t test with the data analysis program StatWorks (Cricket Software, Inc., Philadelphia, Pa.).

**Phenotypic analysis of PBMCs.** PBMCs from healthy donors and HIV-infected individuals, including two patients at stage IV, were analyzed for expression of cell surface markers. After culture for 5 days with or without mycoplasmas, cells were first incubated for 10 min at 4°C with the fluorescein isothiocyanate-conjugated MAb anti-CD3 (SK7, immunoglobulin G1 [IgG1]), anti-CD4 (SK3, IgG1), and anti-CD8 (SK1, IgG1), washed, and then secondarily treated with phycoerythrin-labeled MAb against anti-CD4 or activation markers such as anti-CD69 (L78, IgG1), anti-IL-2 receptor (CD25, 2A3, IgG1), and anti-CD45RA (L243, IgG2a). Anti-human IgG1 (X40) and anti-human IgG2a (X39) were used as negative controls. The MAbS were purchased from Becton Dickinson Immunocytometry Systems (San Jose, Calif.), and optimal concentrations for usage were determined in preliminary experiments. Stained cells were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson) after fixation with 1% paraformaldehyde. Analysis was performed with the software Lysis II (Becton Dickinson).

## RESULTS

**M. penetrans** induces the blastogenesis of lymphocytes from healthy donors and HIV-infected individuals. In vitro coculture of PBMCs from healthy donors with M. penetrans induced, after a few days, the appearance of blast cells and the formation of cell clusters, visible by microscopic examination. The percentage of lymphocyte blast formation after a 5-day culture was determined by FACScan analysis. Blast and nonblast cells were selected by forward-scatter and side-scatter criteria (Fig. 1A). Large blasts appeared in cultures stimulated by mycoplasma in healthy donor PBMCs (mean, 16.7% ± 2.9%) and also in HIV-infected donor PBMCs (mean, 15.1% ± 2.6%), compared with less than 2% of blasts in cultures stimulated with RPMI medium alone (Fig. 1B). PPLO medium containing horse serum used for mycoplasma culture did not induce blastogenesis of cells (less than 2%) (data not shown). Although the degree of blastogenesis induced by M. penetrans was not as high as that induced by SEE (80.6%), the 10-fold increase in blasts induced by M. penetrans when compared with those of unstimulated cultures was remarkable.

**Proliferative response of PBMCs from healthy donors to M. penetrans.** To analyze whether blastogenesis was followed by cell proliferation, the capacity of M. penetrans to induce such a proliferation in PBMCs from healthy donors was tested. Figure 2A shows that M. penetrans added as washed cells induced a significant proliferation of normal PBMCs. When mycoplasmas were added as a cell culture suspension, a proliferative response was also observed, but a peak appeared at 4 days of culture and then decreased. No activity was found associated with the supernatant of M. penetrans culture. The dose dependence of the proliferative response induced by M. penetrans on PBMCs was tested (Fig. 2B). M. penetrans culture added as a suspension at a dose of $0.4 \times 10^6$ CFU/ml induced the highest proliferation of PBMCs. With higher concentrations ($1.7 \times 10^6$ and $0.8 \times 10^6$ CFU/ml), a decreased proliferation was observed after 4 days of culture. Irradiated control PBMCs showed no increase in [3H]thymidine incorporation in the presence of viable mycoplasmas during a 5-day
shown in Fig. 3, M. penetrans can act as a mitogen for PBMCs from both healthy donors and HIV-infected individuals. The level of proliferation in the presence of M. penetrans was significantly different from that observed in unstimulated cultures ($P < 0.0001$). In healthy donors, the response against M. penetrans was variable among different individuals, with ranges of $[^{3}H]$thymidine incorporation between 2,400 and 21,780 cpm; in HIV-infected individuals, the range of $[^{3}H]$thymidine incorporation varied from 420 to 18,540 cpm, indicating that this population included individuals who were nonresponders against M. penetrans. The mean proliferative response to M. penetrans was not statistically different between the two groups (mean of healthy donors, 8,750 ± 1,410 cpm; mean of HIV-infected individuals, 6,227 ± 1,164 cpm). As a positive control in the proliferation experiments, the bacterial superantigen SEE was used. A bacterial superantigen was preferred over other mitogens because its kinetic response was comparable to the one obtained with M. penetrans. The mean proliferative responses to SEE were similar in both healthy donors (mean, 29,161 ± 4,065 cpm) and HIV-positive individuals (mean, 31,043 ± 2,768 cpm). It is noteworthy that two HIV-infected individuals (one stage II and one stage IV) who responded weakly to M. penetrans were also the weakest responders to SEE (less than 2,000 cpm). However, this association between SEE and M. penetrans responses could not be generalized to all of the patients.

Available sera from HIV-infected patients were tested for the presence of antibodies directed against M. penetrans. Of 12 donors tested, only two individuals, who were asymptomatic, showed a strong positive reaction against M. penetrans in the Western blot analysis. Of the two M. penetrans- and HIV-positive individuals, one of them had a high (11,920 cpm) and the other had a low (1,710 cpm) proliferative response to M. penetrans (Fig. 3, asterisks). The small number of M. penetrans-seropositive subjects does not allow us to make a conclusion about an eventual correlation between the presence of M. penetrans-specific antibodies and the magnitude of mycoplasma-induced proliferative response.

M. penetrans induces the expression of activation markers on the surface of T lymphocytes obtained from healthy donors and HIV-infected individuals. Since M. penetrans was able to induce blastogenesis and specific proliferation of PBMCs from healthy donors and HIV-infected individuals, its influence on the expression of activation markers in T lymphocytes was explored. The percentages of cells expressing the activation markers CD69, HLA-DR, and CD25 within the CD3-positive population were measured ex vivo (before in vitro stimulation) and after a 5-day culture in the presence or absence of M. penetrans (Fig. 4). These markers were chosen because CD69 indicates an early activation, HLA-DR indicates a late activation, and CD25 is the receptor for the IL-2 α-chain, p55. Ex vivo, the percentage of CD4+ cells was lower (33.4% ± 11.3% in HIV-positive individuals versus 69.7% ± 7.5% in healthy donors; $P = 0.04$) and the percentage of HLA-DR-expressing cells was higher (10.8% ± 2.0% in HIV-positive individuals versus 1.8% ± 0.9% in healthy donors; $P = 0.01$) in HIV-infected individuals than in healthy donors, as previously reported by others (27). After a 5-day culture with M. penetrans, expression of either CD4 or the activation markers in the CD3+ population was analyzed separately both in nonblast and blast lymphocytes. In healthy donors, the mean CD4+ lymphocyte percentages were similar in unstimulated control cells (71.9% ± 8.4%) and in cells stimulated by M. penetrans (76.9% ± 5.2% for nonblast cells and 63.4% ± 9.2% for blast cells) (Fig. 4A). M. penetrans induced an increased expression of CD69, HLA-DR, and CD25. It is noteworthy that the ex-
pression of CD69 was observed in both blast and nonblast cells in \( M. \) penetrans-stimulated cultures (25.9% ± 4.9% in nonblast cells and 31.1% ± 5.5% in blast cells), suggesting that this marker was expressed before the blastoformation of lymphocytes. On the other hand, HLA-DR and CD25 were expressed at higher levels in blast cells (25.7% ± 5.3% for HLA-DR and 15.5% ± 2.1% for CD25) than in nonblast cells (16.8% ± 7.1% for HLA-DR and 7.6% ± 2.0% for CD25), which is consistent with the fact that these two activation markers tend to be expressed later, after blast transformation. For all three molecules, the increase of their expression in blast cells in \( M. \) penetrans-stimulated cultures was significant in comparison with that in unstimulated cultures.

In HIV-infected individuals (Fig. 4B), the percentage of CD4\(^+\) T lymphocytes among CD3\(^+\) T lymphocytes was not decreased following a 5-day culture, whether stimulated by \( M. \) penetrans or unstimulated. When analyzed, activation markers in HIV-infected individuals displayed the same tendency as those in healthy donors. The increase of CD69 induced by \( M. \) penetrans was significant in both nonblast and blast cells (11.2% ± 3.1% in nonblast cells and 19.8% ± 4.4% in blast cells versus 1.0% ± 0.13% without stimulation). Levels of HLA-DR and CD25 were significantly increased only in blast cells (23.1% ± 3.5% for HLA-DR and 12.5% ± 2.2% for CD25). PBMCs from the two stage-IV patients showed a high expression of HLA-DR without stimulation (11.8 ± 12.1%, respectively). \( M. \) penetrans induced in one of them an increased expression of this marker concomitantly with the induction of CD69 expression (35.8 and 46.3%, respectively, in blastic cells). PBMCs from the other patient responded poorly to \( M. \) penetrans stimulation.

FIG. 3. Comparative proliferative responses induced by \( M. \) penetrans in PBMCs from healthy donors (A) and HIV-infected individuals (B). PBMCs were cultured for 5 days in the presence of \( M. \) penetrans at a concentration of 0.4 \( \times \) 10\(^5\) CFU/ml. SEE at 1 \( \mu \)g/ml was used as a positive control. Statistical analysis was performed by use of Student’s t test, and the P values are shown. The asterisks in panel B indicate two individuals who showed seropositivity to \( M. \) penetrans.

Characteristics of T-cell subsets from healthy donors susceptible to activation by \( M. \) penetrans. The levels of CD69, HLA-DR, and CD25 expression in total lymphocytes (nonblast and blast cells) were analyzed among CD3\(^+\), CD4\(^+\), and CD8\(^+\) subpopulations (Fig. 5). In CD3\(^+\) cells, \( M. \) penetrans induced a significant expression of CD69, followed by HLA-DR and CD25. Furthermore, \( M. \) penetrans was able to activate both CD4\(^+\) and CD8\(^+\) T-cell subsets, the observed differences between activation in the two subsets being not significantly different.

DISCUSSION

In the present study, we have shown the capacity of \( M. \) penetrans, a new species of mycoplasma, to induce in vitro a complete activation of human T lymphocytes characterized by blastoformation followed by cell proliferation and expression of activation markers. This property was reproducible in all of the healthy donors tested, although the level of blastoformation (16%) was lower than that obtained with the superantigen SEE (80%). Kinetics of T-cell proliferation in the presence of \( M. \) penetrans was similar to that obtained by adding SEE to the cells at optimum doses. However, with higher doses of \( M. \) penetrans, T-cell proliferation was reduced, which is probably due to cytotoxic effects of this mycoplasma that have been described by others (18). Among healthy donors, both high and low responders to \( M. \) penetrans stimulation were observed. The high response to \( M. \) penetrans stimulation is not likely due to an antigenic recall response, since the prevalence of \( M. \) penetrans in blood donors, as measured by the level of antibodies, has been found to be lower than 1% (33). However, one cannot exclude the possibility that the serological evaluation of \( M. \) penetrans may not reflect the real prevalence of this mycoplasma, since the detection of \( M. \) penetrans-specific antibodies has still not been correlated with a present or previous infectious process. The stimulation of T lymphocytes induced by \( M. \) penetrans is characterized by the expression of early (CD69) or late (HLA-DR and CD25) markers of activation, the latter suggesting a dependence of T-cell proliferation on IL-2.

Various mycoplasma species, including Mycoplasma ferialis, Mycoplasma pneumoniae, and Mycoplasma arthritidis, have been found to polyclonally activate human T lymphocytes in proliferation assays (1, 2, 4, 28). The present report is the first to show by phenotypic analysis of PBMCs that mycoplas-
FIG. 4. (A and B) M. penetrans-induced expression of activation markers on CD3-positive cells from healthy donors (A) and HIV-infected individuals (B). Activation marker expression after a 5-day culture with and without M. penetrans (0.4 × 10^6 CFU/ml) was analyzed in PBMCs isolated from healthy donors and HIV-infected individuals. Stimulation induced by M. penetrans was analyzed by FACSscan by gating on nonblast or blast cells. In panels A and B, each closed circle corresponds to a single donor; in panel B, open squares and open triangles indicate data for two patients in stage IV (AIDS stage). (C) Activation index induced by M. penetrans in healthy donors and HIV-infected individuals. Expression of CD3, CD4, and activation markers (CD69, HLA-DR, and CD25) was analyzed in total lymphocytes (nonblast and blast cells) in M. penetrans-stimulated or nonstimulated culture. The activation index was calculated as described on the figure.
mas activate both CD4+ and CD8+ human T lymphocytes. The discrepancy with previous results indicating the capacity of cell lysates of *M. pneumoniae*, *Mycoplasma genitalium*, and *M. fermentans* to selectively stimulate CD4+ T lymphocytes (30) may have been caused by differences in the mycoplasma species being studied and in the preparation of stimulants (lysates versus living mycoplasma cells in the present study). However, attempts in our laboratory to specifically activate CD4+ cells with mycoplasma lysates from *M. fermentans* and *M. penetrans* were unsuccessful (data not shown).

Mitogenic activity has been found to correspond to the production of a superantigen, *M. arthritidis* mitogen, which is secreted in the culture medium of this rodent pathogen (4). This is not the case for the mitogenic activity described in the present study, since no activity was found associated with *M. penetrans* culture supernatant. However, it is also known that *M. arthritidis* mitogen is irreversibly inactivated when the pH is below 7.0. Since the pH of the supernatant of an exponentially grown *M. penetrans* culture is between 5.5 and 6.0, a putative superantigen may exist in *M. penetrans* supernatant in an inactive and therefore undetectable form in our biological assay. *M. penetrans*-derived lipid-associated membrane proteins are also mitogenic molecules. Marine B cells have recently been observed to be susceptible to activation with *M. penetrans*-derived lipid-associated membrane proteins (7). The present study indicates that *M. penetrans* activates not only B cells but also T lymphocytes. Bacterial lipoproteins from species as diverse as *Escherichia coli* (29) and *Borrelia burgdorferi* (22) have also been found to be capable of stimulating B lymphocytes. Interestingly, only a weak activation of marine T cells was obtained with *M. penetrans* lipoproteins (7). This discrepancy could be explained in several ways, including differences in the nature of the stimulation (membrane extract versus whole mycoplasma cells) and in the type of cells (mouse versus human). Since preliminary data indicate that *M. penetrans* double-stained PBMCs induces secretion of monokines, including tumor necrosis factor alpha and IL-6, it cannot be excluded that the effects observed in the present study on T lymphocytes might be the consequence of an initial activation of monocytes or macrophages.

Since epidemiological studies have associated the prevalence of *M. penetrans* with HIV infection, the potential role of this mycoplasma as an activator of immune cells from HIV-infected individuals may be important. Our data show that in PBMCs from HIV-infected individuals, *M. penetrans* induced the blastoformation and expression of the activation marker index at a level comparable to that obtained with healthy donors.

Opportunistic infections which can activate the immune system have the capacity to stimulate HIV replication, and the resulting increased viral load correlates with CD4+ lymphocyte depletion and disease progression (9, 15). The pernicious effects of these infections are that they may remain undetected and therefore untreated and that they may be chronic. We believe that these clinically silent infections, by contributing to the activation of the immune system, can have a negative effect on the evolution of HIV disease. This hypothesis deserves further investigation because of its therapeutic implications.

Chronicity is a characteristic of human mycoplasmal infection (3). Data concerning this are still missing for the newly discovered *M. penetrans*, and infection with this mycoplasma in HIV-infected patients has not been associated to date with clinical manifestations (20). Our data, together with those of others (7), indicate the capacity, at least in vitro, of this mycoplasma to stimulate immune cells. This remains to be determined in vivo.

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