

Enhanced Susceptibility of Blood Monocytes from Patients with Pulmonary Tuberculosis to Productive Infection with Human Immunodeficiency Virus Type 1

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Summary

Blood monocytes from patients with active pulmonary tuberculosis and age-matched healthy purified protein derivative-reactive donors were infected with human immunodeficiency virus type 1 (HIV-1)_{JR-FL} in vitro to assess their susceptibility to productive infection by HIV-1. HIV-1 p24 levels (enzyme-linked immunosorbent assay) in supernatants of infected cells from patients with tuberculosis, albeit variable, were significantly higher at days 10–20 of culture; the maximum levels of p24 antigen were greater in supernatants of HIV-1-infected monocytes from patients than maximum levels for controls ($p < 0.05$). The maximum increment in p24 levels for patients also exceeded that for controls ($p < 0.05$). Entry of HIV-1 and/or initiation of reverse transcription, measured by polymerase chain reaction using HIV-1 R/U5 primer pairs, was variable and low in infected monocytes from both patients and controls, and did not correlate with HIV-1 p24 levels. The frequency of infected cells as assessed by endpoint dilution viral cultures was similar for both groups. Therefore, blood monocytes from patients with active tuberculosis can develop a highly productive infection with HIV-1 that does not appear to be due to enhanced HIV entry or higher frequency of infected cells. The enhanced susceptibility may result directly from activation of monocytes by exposure to *Mycobacterium tuberculosis* and its products in situ.

Infection with HIV-1 appears to be responsible for the recent rise in the prevalence of tuberculosis in the United States (1) and in developing countries (2–4). Among individuals infected with *Mycobacterium tuberculosis*, HIV-1 infection is the greatest known risk factor for development of active tuberculosis (5, 6). Furthermore, HIV-1-infected individuals have a high likelihood of developing progressive tuberculosis upon new infection with *M. tuberculosis* (7).

The impact of tuberculosis on HIV-1 disease is beginning to be recognized both in the United States (C. Whalen, personal communication) and in other countries (8–10). Since tuberculosis tends to occur early in the course of HIV-1 disease, the interaction of the two disease processes may have a pronounced effect on viral burden and may facilitate the progression of HIV-1 disease to advanced stages. Active tuberculosis in the HIV-1-seronegative patient is associated with several stigmas of activation of the immune system that may be conducive to enhancement of viral activity. Blood monocytes from patients with tuberculosis express markers of activation such as surface IL-2R (11) and production of TGF- β (12). Furthermore, upon in vitro stimulation with LPS and

proteins of *M. tuberculosis*, monocytes from patients release increased amounts of TNF- α (13), IL-1 (14), and IL-6 (13). Studies of mononuclear cells from HIV-1-infected patients with tuberculosis also show enhanced purified protein derivative (PPD)-stimulated TNF- α production at the time of diagnosis of tuberculosis (10). IL-1, IL-6, and TNF- α , in turn, are capable of enhancing HIV-1 production at several stages of viral replication (15). Since circulating monocytes from tuberculous subjects appear to be primed in vivo with regard to a variety of functions, we postulated that these cells may display an enhanced in vitro susceptibility to infection with HIV-1.

Materials and Methods

Study Subjects. Patients with newly diagnosed active pulmonary tuberculosis receiving care at Cleveland Veteran's Administration Medical Center, University Hospitals of Cleveland, and Cleveland MetroHealth Medical Center were recruited for study. The exclusion criteria were: >65 yr of age; antituberculous therapy for >6 wk; concomitant debilitating illness, including malignancy, di-

abetes, and other serious medical conditions; and positive serology for HIV-1. Diagnosis of tuberculosis was based on the demonstration of acid-fast bacilli in sputum and was confirmed by a positive culture for *M. tuberculosis*. Each tuberculous patient was studied along with a healthy, HIV-1-negative, and tuberculin-reactive age-matched subject. Two individuals with pulmonary processes other than tuberculosis also were studied. One had a granulomatous disease secondary to *Histoplasma capsulatum*. The second patient had an atypical lower lobe noncavitary pneumonia and was tuberculin reactive. Both patients were subjected to the same exclusionary criteria as patients with tuberculosis and were studied along with a healthy tuberculin-reactive subject. None of the patients had fever at the time of study. All had hematocrits >30, and serum albumins >3.2 g/dl. This protocol was approved by the Institutional Review Boards of the above hospitals.

Preparation of Cells. PBMC were isolated from 30 ml of heparinized blood (20 U/ml) by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradients (11). To obtain monocytes and nonadherent (NA) cells, PBMC were resuspended at 5×10^6 cells/ml in RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) with 10% (vol/vol) of heat-inactivated pooled human serum (PHS), and incubated on plastic petri dishes that were precoated with 1 ml of PHS. After 1 h plates were vortexed, NA cells were aspirated, and plates were washed with prewarmed RPMI 1640 with 10% (vol/vol) FCS (HyClone Laboratories, Logan, UT). Adherent cells (ADH) were 90–95% monocytes by peroxidase cytochemistry (11). NA cells were 70–80% T cells (OKT3 reactive) and contained up to 5% peroxidase-reactive cells (monocytes). To further deplete NA cells from ADH, the ADH were further incubated in RPMI 1640 with 10% (vol/vol) PHS overnight and monolayers were washed before infection with HIV-1 as described previously (16). This population was 99% peroxidase reactive and all monocytes by morphology (Wright cytochemistry), and was >98% viable by trypan blue staining.

Infection of Cells with HIV-1. The viral isolate used in this study was HIV-1_{JR-FL}, which was recovered from the frontal lobe brain of a patient with AIDS (16). This strain replicates in both activated lymphocytes and mononuclear phagocytes. Viral stock was prepared from 8-d culture supernatants of infected PHA-stimulated PBMC, quantitated for HIV-1 p24 antigen by ELISA, and the stocks were stored at -70°C . By limiting dilution assay on PHA-stimulated PBMC, ~ 30 pg of p24 was equivalent to one infectious unit of HIV-1. Before use, to deplete contaminating cell-associated proviral DNA, each aliquot of viral stock was filtered through a 0.22- μm filter (Gelman Sciences Inc., Ann Arbor, MI) and was treated with 1.8 $\mu\text{g}/\text{ml}$ of RNase-free DNase (Worthington Biochemical Corp., Freehold, NJ) in the presence of 10 μM of MgCl_2 . Cells were infected using the treated viral stock (400 ng p24/ml) together with polybrene (5 $\mu\text{g}/\text{ml}$) and were cultured for 2 h at 37°C . Duplicate cultures received heat-inactivated (HIA) virus (viral stock heated for 1 h at 56°C) that had been DNase treated and filtered as had the live virus. After incubation, virus-containing medium was aspirated and discarded, and cells were washed twice with PBS. To remove cells, PBS containing 0.5 mM EDTA was added to plates for 10 min at room temperature. Then cells were scraped gently, sedimented at 1,500 rpm for 5 min, and resuspended in Iscove's medium (BioWhittaker, Inc.) containing 15% (vol/vol) PHS and 5% (vol/vol) FCS (complete Iscove's). Cells were counted and plated at $5 \times 10^5/0.5$ ml of complete Iscove's in wells of 24-well plates. Viability was assessed by trypan blue and was 99%.

Detection of HIV-1 p24 Antigen by ELISA. Culture supernatants were collected every 4 d up to 20 d after infection with HIV-1. The medium was changed 24 h before each collection. An enzyme-

linked immunoassay kit for HIV-1 p24 antigen (Coulter Immunology, Hialeah, FL) was used. This assay is sensitive to 7.5 pg/ml of p24 antigen.

HIV-1 Detection by PCR. After infection, monocytes were incubated at $5 \times 10^5/0.5$ ml of complete Iscove's for 24 h at 37°C . The medium was removed and cells were washed once with PBS, and then lysed with 100 μl of lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl_2 , 0.1 mg/ml gelatin [Sigma Chemical Co., St. Louis, MO], 0.45% NP-40, 0.45% Tween 20, and 1 mg/ml proteinase K [Boehringer Mannheim Biochemicals, Indianapolis, IN]) (16). The cell lysates were incubated at 60°C for 1 h to allow lysis of proteins by proteinase K and then at 95°C for 10 min to inactivate the proteinase K, and stored at -70°C until use.

The amplification reaction was carried out as described previously (18). The 5' sense oligonucleotide primer for each pair was end labeled with γ - ^{32}P ATP (ICN Laboratories, Irvine, CA). Each reaction contained 10 μl of sample, 1.66 U of taq polymerase (Boehringer Mannheim Biochemicals), 50 ng of each HIV-1 primer, 0.25 mM of each of the four dNTPs, 50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 5 mM MgCl_2 , and 0.1 mg/ml BSA. To detect β -globin sequences, 25 ng of labeled sense primer plus 25 ng of unlabeled antisense primer was used to reduce the intensity of the signal. The total volume of the reaction mixture (25 μl) was overlaid with one drop of mineral oil and subjected to 25 cycles of denaturation at 94°C for 1 min and annealing and polymerization at 65°C for 2 min. The primers used to detect HIV-1 sequences were M667/AA55, which amplifies a conserved region in the HIV-1 LTR (R/U5) 140 of bp (18). The nucleotide sequences are: M667, 5'-GCTAACTAGGGAACCCA CTG-3'; AA55, 5'-CTGCTAGAG-ATTTTCCACACTGAC-3'. Amplification of β -globin sequences was performed to normalize the amount of DNA content between samples. A primer pair that amplifies a 110-bp product in the first exon of the human β -globin gene was used for this purpose (18). The sequences are 5'-ACACAACCTGTGTTCACTAGC-3' sense and 5'-CAACTTCATCCACGTTCCACC-3' antisense. The amplified products were separated electrophoretically on a nondenaturing polyacrylamide gel. The gel was dried and exposed to radiographic Kodak XAR-5 film for various periods of time at room temperature.

To determine the number of copies of HIV-1 present in the amplified product of each reaction, known copy numbers of HIV-1 DNA were prepared from cloned HIV-1_{JR-CSF} DNA (pNB_{JR-CSF}) linearized with EcoRI, which does not cleave viral sequences. HIV-1 standards of 3–3,000 copies were amplified concurrently with samples in each experiment. HIV-1-negative PBMC DNA lysed with the same lysis buffer as test samples was used to prepare DNA standards (30–1,000 ng), and these standards also were amplified concurrently with samples.

Endpoint Dilution Viral Culture. The titers of infectious HIV-1 of in vitro infected monocytes were determined using the endpoint dilution assay as described by Ho et al. (19). PBMC from a seronegative donor were stimulated with PHA (1 $\mu\text{g}/\text{ml}$) for 2 or 3 d and washed. The PHA-stimulated PBMC then were suspended at 2×10^6 cells/ml in growth medium that consisted of RPMI 1640, 20% FCS, 10% IL-2 (Cellular Products, Buffalo, NY), 2 μM L-glutamine, 5 $\mu\text{g}/\text{ml}$ gentamicin, and 50 U/ml penicillin. 1 ml of PBMC was placed in each well of a 24-well tissue culture plate. The monocytes were suspended in growth medium in fivefold dilutions from 125×10^3 to 0.2×10^3 cells/ml. 1 ml of each dilution of the adherent cells was added to the PHA-stimulated PBMC. One-half of the medium was changed twice per week with fresh growth medium without disturbing the cells at the bottom of the wells. Supernatants were collected every 1 wk for 4 wk and assayed

for HIV-1 p24 antigen by ELISA. A culture was considered positive when the p24 antigen was 100 pg/ml or higher. The lowest dilution with a positive culture was considered the endpoint and the results were expressed as tissue culture infective dose (TCID) $_{50}$ /125 \times 10³ cells.

Statistical Analysis. All HIV-1 p24 values were logarithmically transformed, and paired *t* tests were performed between each patient control group.

Results

HIV-1 Infection of Monocytes from Patients with Tuberculosis. Monocytes from five patients with tuberculosis and from five age-matched healthy individuals were infected with HIV-1_{JR-FL} as described in Materials and Methods. Supernatants were collected for a 24-h period every 4 d after infection and assayed for HIV-1 p24 by ELISA. Each panel in Fig. 1 represents one patient-control pair studied. After day 8, patients had significantly higher values of p24 in supernatants at several time points: day 12, *p* < 0.02; day 16, *p* < 0.05; and day 20, *p* < 0.05. Levels of p24 in supernatants of monocytes from patients with tuberculosis were <2–60-fold higher than controls at day 12. The maximum p24 values in monocyte supernatants from patients were consistently greater than the maximum values for paired controls (*p* < 0.05). The maximum increments in p24 values during culture also were greater for patients than controls (*p* < 0.05). As seen in Fig. 1, the degree of support of HIV production was variable among the patients. The maximal viral activity detected in supernatants of monocytes from the patients over control subjects in Fig. 1, A (50-fold) and C (over 1000-fold), was higher as contrasted with those in Fig. 1, B (3.4-fold), D (2-fold), and E (1.6-fold). However, even when the high producers were excluded from the analysis, the differences in HIV p24 levels of supernatants of monocytes from patients with tuber-

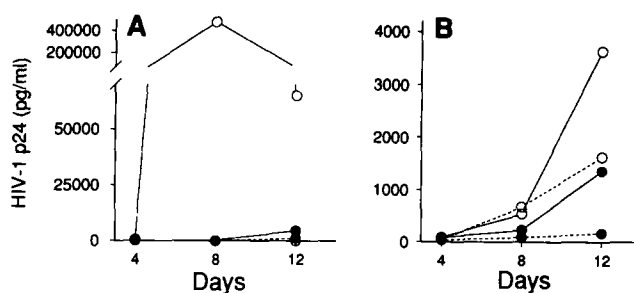


Figure 2. HIV-1 p24 levels (in pg/ml) in supernatants of monocytes and NA cells. Monocytes and NA cells from each patient-control (A and B) pair were infected with HIV-1_{JR-FL} and culture supernatants were assessed for p24 antigen produced over 24 h every 4 d by ELISA. Patient monocyte (O—O); patient NA cell (O—O); control monocyte (●—●); control NA (●—●).

culosis and healthy individuals remained significantly higher on day 16 (*p* < 0.05) and day 20 (*p* < 0.02).

Since HIV-1_{JR-FL}, although monocytophilic, also can infect activated primary T cells (18), it was necessary to assure that the high viral titers in cultures of infected monocytes from patients were not a reflection of infected activated lymphocytes potentially contaminating preparations of monocytes. Therefore, in two patient-control pairs, NA cells, which are ~70–80% T lymphocytes and contain up to 5% monocytes, were infected in vitro with HIV-1_{JR-FL} in parallel with monocytes. Viral activity was assessed in NA culture supernatants at various periods thereafter by ELISA for p24 antigen. As shown in Fig. 2, viral activity in supernatants of NA cells was less than or equal, and never higher than supernatants of monocytes. These data indicate that the p24 antigen content of monocyte cell supernatants is not a result of infected NA cells within this population.

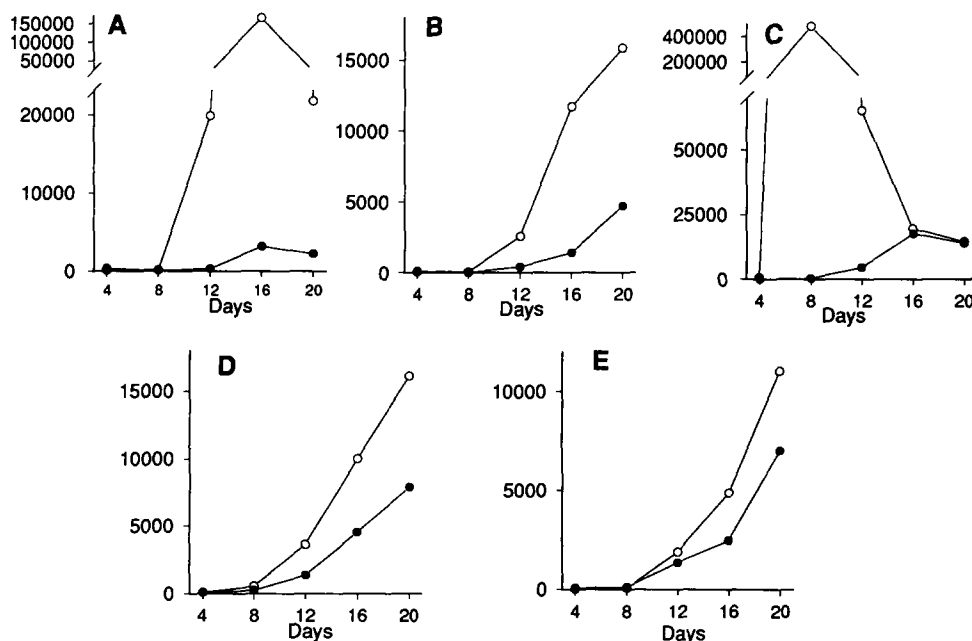


Figure 1. HIV-1 p24 levels (in pg/ml) in supernatants of monocytes from patients with tuberculosis and healthy control subjects. Monocytes from each patient-control pair were infected with HIV-1_{JR-FL} and culture supernatants were assessed for p24 antigen produced over 24 h every 4 d by ELISA. Each panel demonstrates results for one patient-control pair. Patient (O); control (●).

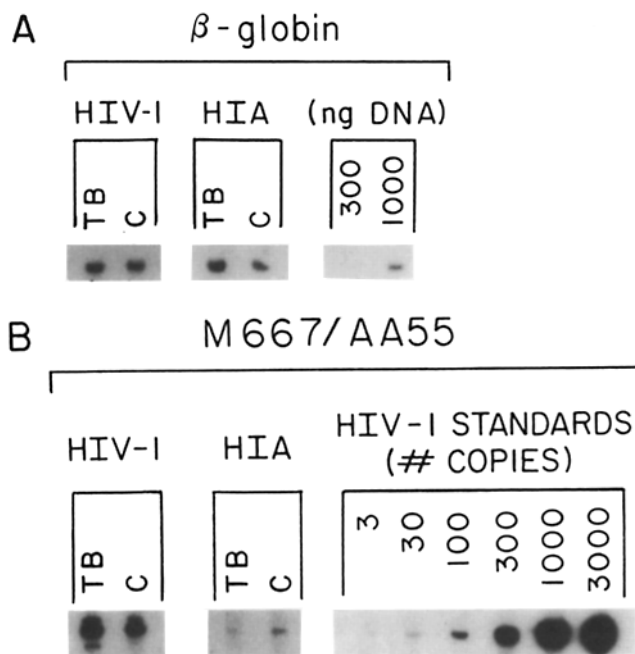


Figure 3. Quantification of HIV-1 DNA in monocytes from patients with tuberculosis and healthy control subjects by PCR. Monocytes (10^4 cells) from a patient (TB)-control (C) subject pair were infected with HIV-1_{JR-FL}, or HIA virus. At 24 h after infection, cells were lysed and DNA amplification was performed using primer pairs specific for β -globin (A), and for the HIV-1 R/U5 region using M667/AA55 primer pairs (B). Amplified products were resolved onto a gel and autoradiography was performed. Samples and known amounts of PBMC DNA were amplified concurrently using the β -globin primers to quantify cellular levels of DNA in the samples (A). Samples and known copy numbers of HIV-1 DNA were amplified concurrently with the R/U5 primers to quantify copies of HIV-1 (B).

To assess if pulmonary processes other than tuberculosis increased the susceptibility of monocytes to productive infection with HIV-1, monocytes from a patient with pulmonary histoplasmosis, a patient with atypical pneumonia, and a healthy control subject were infected with HIV-1_{JR-FL}; culture supernatants were assessed for HIV-1 p24 levels over 24 h every 4 d, up to 20 d. The level of HIV-1 p24 culture supernatants of monocytes from both patients remained lower than the healthy individual. Maximal levels were reached for each subject on day 12 of culture, and were 1,756 pg/ml for the patient with histoplasmosis, 3,038 pg/ml for the patient with atypical pneumonia, and 13,000 pg/ml for the control subject.

HIV-1 DNA Synthesis in Monocytes from Patients with Tuberculosis. Enhanced HIV-1 production in monocytes from tuberculous subjects could potentially be a result of early events in viral DNA synthesis after infection. To assess this possibility, we examined the load of HIV-1 DNA in the monocytes after infection. A semiquantitative PCR was performed on infected adherent cells using primer pairs (M667/AA55) that flank the start site of reverse transcription in the R/U5 region of the LTR, and therefore detect all, including incomplete, HIV-1 reverse transcripts. Monocytes from each patient-control pair (Fig. 1) were infected with HIV-1_{JR-FL}

or with virus that was heat inactivated. 24 h after infection, cell lysates were prepared as described in Materials and Methods for PCR analysis. The amount of total cellular DNA was quantified in each sample using human β -globin-specific oligonucleotide primers. Concurrently, an equal volume of cell lysate was subjected to PCR using M667/AA55. A representative experiment is shown in Fig. 3; both patient and control had 300–1,000 copies of HIV-1/ 10^4 cells. The autoradiograms of the amplified HIV-1 R/U5 and globin products were analyzed by laser densitometry, and data for each patient-control pair were normalized to the densitometric readings of the globins. In four of five experiments HIV-1 R/U5 product was higher in patients than controls (mean, 2.1-fold) (Table 1). In one experiment HIV-1 R/U5 product was 1.9-fold higher in the control subject. As seen in Table 1, however, there was no correlation between the HIV-1 R/U5 data and HIV-1 p24 antigens in supernatants; the correlation coefficient for p24 levels and the HIV-1 R/U5 product was $r = -0.05$. These data indicated that viral entry and/or early events in reverse transcription in monocytes were generally higher in the monocytes from patients but were insufficient to explain the difference in the capacity to support a productive viral infection.

Frequency of Infected Monocytes. To determine if the higher production of HIV-1 by monocytes from patients with tuberculosis could be attributed to an increased frequency of infected cells, endpoint dilution viral cultures were performed. Monocytes from patients and healthy controls (Fig. 1, A, C, and E) were infected with HIV-1_{JR-FL}. Immediately after infection, fivefold serial dilutions of washed monocytes from patients and healthy individuals were cultured with PHA-stimulated PBMC in duplicate. To assure no carryover of free virus, the media from the last cell wash (postinfection) were cultured with PHA-stimulated PBMC. HIV-1 p24 antigen in culture supernatants was assessed weekly (up to 4 wk) and the frequency of HIV-1-positive cells calculated as TCID₅₀/125 $\times 10^3$ cells. In each of the three experiments performed, frequency of infected cells in the adherent cells ranged from 25 to 125 TCID₅₀/125 $\times 10^3$ and was similar in the patient-control pairs. HIV-1 p24 antigen was undetectable in the wash media.

Discussion

Blood monocytes from patients with active newly diagnosed pulmonary tuberculosis were more susceptible to a productive infection with HIV-1_{JR-FL} as compared with monocytes from healthy PPD-reactive individuals. Whereas differences in monocyte susceptibility to HIV-1_{JR-FL} infection between some patients and concurrent control subjects were very high (50–1,000-fold), in others these differences were more modest (mean, 2.3-fold). The enhanced viral expression by monocytes from patients was not due to a higher frequency of cells infected with HIV-1 during the primary infection or to early events after infection, including initiation of reverse transcription.

Table 1. Relationship between HIV-1 p24 Levels and Determination of Reverse Transcription of HIV-1 in Infected Monocytes

Exp.	HIV-1 p24 on day 12		HIV-1 R/U5	
		pg/ml	densitometric units*	
A Patient		19,830	1,010	1.6 [§]
		59.3 [‡]		
Control		334	600	
B Patient		2,570	3,828	4.1
		6.2		
Control		413	922	
C Patient		65,180	2,500	1.4
		14.0		
Control		4,647	1,699	
D Patient		3,635	2,297	1.4
		2.7		
Control		1,351	1,639	
E Patient		1,894	1,520	0.52
		1.4		
Control		1,355	2,912	

* Autoradiograms of the amplified HIV-1 R/U5 and globin products were analyzed by laser densitometry, and the area under curve was ascribed arbitrary densitometric units. In each patient-control experiment the globin readings were normalized to the globin reading from the control and R/U5 densitometric readings corrected accordingly.

‡ HIV-1 p24 in monocyte culture of patient/HIV-1 p24 in monocyte culture of control.

§ HIV-1 R/U5 densitometric value in monocytes from patient/HIV-1 R/U5 densitometric value in monocytes from control.

The states of differentiation and activation of mononuclear phagocytes are determinants of infectability with HIV-1. Both alveolar macrophages, which are tissue macrophages differentiated from blood monocytes and activated in vivo presumably by chronic exposure to inhaled antigens, and in vitro cultured monocytes from healthy subjects support a productive infection with HIV-1_{JR-FL}, whereas freshly isolated monocytes develop a minimally productive infection (16). In the current study, undifferentiated primary blood monocytes from healthy subjects again supported a low-level infection with HIV-1. Blood monocytes from tuberculous subjects appear to be activated in vivo because a high percentage express IL-2R (11), produce TGF- β (12), and display high levels of intracellular enzymatic activity (20). Furthermore, monocytes from patients with active tuberculosis are primed to increased production of cytokines upon in vitro stimulation (13, 14). Therefore, the increased susceptibility of monocytes from patients with active pulmonary tuberculosis to productive in-

fection with HIV-1_{JR-FL} in vitro may be a consequence of their activated state and may be mediated by cytokines. On the other hand, blood monocytes from a patient with newly diagnosed active pulmonary histoplasmosis and from a patient with atypical pneumonia did not support viral expression in vitro, suggesting a specificity to the observation during tuberculosis. The nature and abundance of mycobacterial protein and lipid antigens to which mononuclear cells are exposed in situ may underlie the observed differences in HIV-1 susceptibility of mononuclear phagocytes during tuberculosis as compared with other pulmonary processes. The variability in support of HIV production by monocytes from patients with active tuberculosis, however, may relate to differences in their state of activation before HIV infection, and may parallel the variations in other markers of activation, such as IL-2R expression (11). In the infected cell many of the stages of HIV life cycle, such as completion of reverse transcription, integration within the host genome, and viral transcription, are dependent on the degree of cellular activation. Therefore, it is possible that in some patients, one or several of these stages are affected. Whether monocytes from patients with tuberculosis also are more permissive targets to HIV strains other than macrophage-tropic strains, such as HIV-1_{JR-FL}, has not been studied.

The increased viral expression in monocyte cultures from patients with tuberculosis was not due to contamination by T lymphocytes. Morphologically, T cells were not detected in monocyte populations prepared by overnight adherence before infection. Furthermore, T lymphocytes require activation by antigenic or mitogenic stimuli in vitro for a productive infection by HIV-1 to ensue (18). Since our cultures were not stimulated in vitro, it is therefore particularly unlikely that our results can be attributed to infection of T lymphocytes with HIV-1. Also, the differences in p24 activity of NA cell cultures from patients and healthy controls in the present study did not reflect differences in p24 activity of monocyte supernatants from patients and control subjects.

The basis for the increased productive infection of monocytes from patients with active pulmonary tuberculosis with HIV-1 was examined. There was no difference in the frequency of infected monocytes from patients and healthy controls. Differences observed in the number of copies of HIV-1 in infected monocytes from patients and controls using R/U5 primer pairs that detect all initiated, including incomplete, intermediate and complete reverse transcripts were variable and did not correlate with HIV-1 p24 levels in supernatants. Amplified products detected by this primer pair correlate with entry of virus in T lymphocytes (18). Thus, the increased productive infection by monocytes from patients with tuberculosis could not be attributed entirely to early events after infection, including HIV-1 entry or initiation of reverse transcription. We are presently investigating if the basis for enhanced production of HIV-1 in monocytes from patients with tuberculosis is related to the increased production of or response to proinflammatory cytokines in these cells.

Our in vitro findings of enhanced susceptibility of monocytes from patients with tuberculosis to productive infection

with HIV-1 may indicate that, in the individual with dual infection (i.e., HIV-1-seropositive patient who develops tuberculosis), a vast augmentation of viral load could occur both in the blood cells and within tissues. We further speculate that the consequences to the dually infected host may be acceleration to AIDS and ultimately death. Future studies may correlate the association of excess production of proinflammatory

cytokines with enhanced viral production in monocytes from patients with tuberculosis that are infected with HIV in vitro or in vivo. Trials of agents that block TNF- α (21, 22), in particular those that block TNF- α more selectively, such as thalidomide (22), may prove particularly useful in dually infected patients.

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