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*J Immunol* 1999; 163:1506-1515;
http://www.jimmunol.org/content/163/3/1506

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Infection of HIV-1 Transgenic Mice with Mycobacterium avium Induces the Expression of Infectious Virus Selectively from a Mac-1-Positive Host Cell Population

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Infection of HIV-1-transgenic mice with Mycobacterium avium, a common opportunistic pathogen in AIDS patients, was shown to result in increased tissue expression of viral specific transcripts. Moreover, by coculturing splenocytes from the transgenic animals with human T cells it was possible to demonstrate that the elevation in HIV-1 mRNA triggered by M. avium infection reflects increased production of infectious virions. Viral immune activation was also shown to correlate with a marked elevation of p24 in supernatants of ex vivo-cultured tissues and, more importantly, in systemic increases in the HIV-1 protein in plasma. Interestingly, these tissue and systemic p24 responses were found to be differentially regulated. Thus, while in vitro p24 production by cultured splenocytes increased concurrently with bacterial loads during the first 6 wk of infection, levels of the Ag in plasma actually decreased. In situ localization experiments together with FACS analysis of HIV-1-expressing splenocytes indicated that virus production is restricted largely to cells of the monocyte/macrophage lineage. Indeed, in vitro p24 expression by cells from noninfected transgenic mice was up-regulated by polyclonal stimulation of macrophages but not T cells. Together these results underscore the importance of the macrophage reservoir in persistent virus expression and establish a convenient and relevant animal model for studying the factors responsible for immune activation of HIV-1 induced by mycobacterial as well as other common coinfections encountered by AIDS patients. The Journal of Immunology, 1999, 163: 1506–1515.

Immune activation by common infectious and/or opportunistic pathogens has been postulated to be one of the cofactors determining the rate and severity of disease progression in HIV-1-infected (HIV+1) individuals and is frequently cited as a probable explanation of the rapid course of AIDS in Sub-Saharan Africa and other developing regions (1–3). Intracellular bacterial and protozoan infections in particular have been associated with AIDS progression since HIV+ patients show diminished resistance to many of these pathogens, which themselves have been shown to be potent activators of virus expression in vitro. This positive feedback relationship between coinfection and HIV has been most thoroughly documented in the case of Mycobacterium tuberculosis, a bacterial pathogen frequently associated with AIDS in Africa (4) as well as in HIV+ individuals of lower socioeconomic status in developed countries. During acute infection of HIV+ subjects with M. tuberculosis, marked increases in plasma viremia are observed, increases that return to baseline following successful antmycobacterial therapy (5). Moreover, M. tuberculosis+ HIV+ individuals appear to display a more accelerated AIDS progression (6), particularly in terms of increased susceptibility to unrelated opportunistic infections (7). Although other intracellular opportunistic pathogens in addition to M. tuberculosis are likely to promote the progression of AIDS, such associations may be more difficult to document since many of these agents (e.g., M. avium, Toxoplasma gondii, and Histoplasma capsulatum) emerge late in HIV-1 infection when CD4+ counts have already dropped substantially and AIDS is fully developed.

The above-mentioned intracellular pathogens are activators of CD4+ T lymphocyte function and, as a result, could promote HIV-1 expression by enhancing viral infectivity, reverse transcription, integration, and spread within newly infected cells (1, 8). Perhaps more importantly, these microbial agents are strong inducers of many of the proinflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) known to stimulate transcription, assembly, and HIV-1 release within already infected cells (9). In the case of TNF-α, HIV-1 transcription is enhanced through the induction of NF-κB, which interacts with promoter sequences within the long terminal repeat (LTR) of the HIV-1 genome (10). Mycobacteria and other intracellular pathogens are potent inducers of NF-κB activity (11, 12), and, thus, coinfection with these microbes could drive HIV-1 expansion in chronic AIDS patients.

In considering the effects of microbial coinfection on HIV-1 expression, a critical question concerns the cellular source of the virus induced. Persistently infected memory T cells carrying integrated provirus represent one potential source susceptible to NF-κB-dependent immune activation (9). Cells of the monocyte/macrophage lineage may also provide an important reservoir for viral induction, particularly in the latter stages of the disease when CD4

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Received for publication March 10, 1999. Accepted for publication May 24, 1999.

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Abreviations used in this paper: LTR, long terminal repeat; HPRT, hypoxanthine phosphoribosyltransferase; ISH, in situ hybridization; FISH, fluorescence in situ hybridization; SAC, Staphylococcus aureus Cowan.
lymphocyte counts are greatly reduced (13). HIV-1 infection of macrophages is typically noncytopathic, and cells of this lineage have a long 14-day half-life (14). In addition, in vitro virus production by HIV-1-infected macrophages has been shown to be markedly up-regulated as a result of exposure to microbial stimuli, with the NF-κB pathway of transcriptional regulation again playing a major role (1, 9, 11, 13). That macrophages can serve as a major source of latent virus expression has been confirmed recently by in situ localization of virus in tissues of AIDS patients exposed to a bacterial opportunistic infection (15, 16).

We have been developing an in vivo experimental model, which employs transgenic mice that carry complete DNA copies of the HIV-1 genome, to study the mechanism(s) whereby coinfections induce HIV-1 expression in latently infected hosts. The tissues of these animals produce low levels of viral mRNA, as well as infectious virus recoverable by coculture with human T cells (17). Since no viral spread occurs in these mice because of the lack of the appropriate receptors and coreceptors, they are of limited value for the study of HIV infectivity or AIDS pathogenesis. Nevertheless, because of the presence of integrated provirus, including an unaltered LTR, the animals can be used to focus on the factors that regulate latent viral expression. Our initial work using this model (12) showed that infection of line 166 transgenic mice with T. gondii, an opportunistic intracellular protozoan parasite that stimulates a strong proinflammatory and Th1-biased immune response (18), results in elevated HIV-1 gene expression in tissues invaded by the pathogen. The latter response was not seen in an equivalent transgenic mouse line in which the NF-κB-binding regions of the proviral LTR were genetically inactivated.

In the present study, we have extended our analysis of coinfection-induced HIV-1 expression in the transgenic mouse model by demonstrating that the increased proviral mRNA observed is reflected in the enhanced synthesis of viral protein (p24), as well as the assembly and release of infectious virions. Moreover, we show that a second intracellular opportunistic pathogen, Mycobacterium avium, can also serve as potent microbial stimulus of both localized and systemic HIV-1 expression and demonstrate that virus levels change during the course of infection of line 166 mice with this bacterial agent. Finally, by means of in situ as well as in vitro analyses, we establish that cells of the monocyte/macrophage lineage are the major and selective source of virus production in animals exposed to the above microbial stimuli. Taken together, our findings support the concept that macrophages containing integrated proviral DNA can serve as an important reservoir for microbially induced immune activation and establish a useful and relevant in vivo model for studying this process in the context of mycobacterial infection.

Materials and Methods

Animals

The transgenic mouse line 166 was derived as previously described (12, 19) by pronuclear injection of FVB/N mouse embryos with proviral DNA encoding the entire genome of the NL4-3 molecular clone, a T cell-tropic strain of HIV-1. The resulting animals contained 20–60 copies of the proviral transgenes present at single integration sites and transmitted them in a stable Mendelian fashion. Mice were maintained by homzygous breeding under specific pathogen-free conditions in an escape-proof facility within the animal care facilities of the National Institute of Allergy and Infectious Diseases (NIAID, Bethesda, MD). Nontransgenic FVB/N mice were bred in the same facility as controls. Age- and sex-matched transgenic and control animals between 6 and 12 wk old were used in all experiments.

Infectious agents

The M. avium strain 2-151 SmT was kindly provided by Dr. A. Cooper, Colorado State University. Stocks were prepared containing 10^9 CFU/ml in saline, as previously described (20), and stored frozen at −70°C. The avirulent ME-49 strain of T. gondii was maintained by passage in mice, as previously described (21), and infectious tissue cysts were derived from the brains of animals infected at least 1 mo previously. Tachyzoites of the temperature-sensitive T.gondii strain of T. gondii were obtained by tissue culture in human fibroblasts utilizing published procedures (22).

Experimental infections

For infection with M. avium, mice were each injected i.v. using a dose of either 10^3 or 10^4 CFU (suspended in PBS) in a total of 300 μl. To assess bacterial load, spleens were harvested at various time points postinfection, and single cell suspensions were prepared and serially diluted from 10^9 to 10^0 CFU/ml in buffered saline and cultured on agar plates on Sabouraud’s agar as previously described (20). CFU per 10^6 cells were translated to CFU per spleen by multiplying by the total splenocyte count. For infection with T. gondii, mice were inoculated i.p. with 30 brain-derived cysts.

Measurement of HIV-1 gene expression by RT-PCR

Relative levels of HIV-1 mRNA were determined by RT-PCR using an adaptation of a previously described protocol (23). Briefly, single cell suspensions prepared from spleen and other tissues were thoroughly washed, and total RNA was prepared by lysis of 10^6 cells in RNA-STAT-60 (Tel-Test, Friendswood, TX), followed by precipitation from the aqueous phase as recommended by the manufacturer. Recovered RNA was resuspended in diethyl pyrocarbonate-treated, distilled, deionized water, and cDNA was synthesized. PCR reactions were performed on serial dilutions of cDNA (from 10 μl) in a final volume of 50 μl, and a sample (10 μl) of each PCR reaction was electrophoresed through a 1.0% agarose gel and visualized with ethidium bromide. The number of cycles of PCR amplification used was first determined by amplifying cDNA through 24 to 36 cycles and comparing the product obtained to a standard curve from LPS-stimulated spleen cells. The number of cycles of amplification was chosen to give a PCR product that was easily detected in a gel, while remaining on the linear part of the amplification curve. Ethidium bromide-stained gels were photographed with an Eagle Eye II Still Video System (Stratagene, La Jolla, CA), and the intensity of fluorescence was determined using the associated Eaglesight software. To ensure that equivalent amounts of cDNA were used in each reaction, PCR was performed for hypoxanthine phosphoribosyltransferase (HPRT) from each sample, and the cDNA was adjusted to equivalent levels. Both pairs of primers (which were synthesized at the NAID) spanned at least one intron, allowing mRNA to be distinguished from the appropriate receptors and coreceptors, they are of limited value for the study of HIV infectivity or AIDS pathogenesis. Nevertheless, because of the presence of integrated provirus, including an unaltered LTR, the animals can be used to focus on the factors that regulate latent viral expression. Our initial work using this model (12) showed that infection of line 166 transgenic mice with T. gondii, an opportunistic intracellular protozoan parasite that stimulates a strong proinflammatory and Th1-biased immune response (18), results in elevated HIV-1 gene expression in tissues invaded by the pathogen. The latter response was not seen in an equivalent transgenic mouse line in which the NF-κB-binding regions of the proviral LTR were genetically inactivated.

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Detection of infectious virions by in vitro culture in MT4 cells

The presence of infectious virions in transgenic mouse tissue was assessed by coculture with a human T lymphocytic line. Spleen cells were harvested from uninfected or T. gondii- or M. avium-infected line 166 mice, or, as controls, FVB/N mice infected with the same agents. The splenocytes were then distributed in 24-well Costar chambers (Corning Costar, Cambridge, MA) in 500-μl volumes of RPMI 1640 (Life Technologies, Grand Island, NY), 10% FCS (HyClone, Logan, UT), 50 μM penicillin (National Institutes of Health (NIH) stock), 50 μM streptomycin (NIH stock), and 2 mM glutamine (NIH stock). Log phase cells of the human T cell line MT-4 (24) were added to each well in 500 μl of culture medium at 2 × 10^5 cells/ml, and culture supernatants were assayed every 2 days for the presence of reverse transcriptase as described previously (25). Briefly, 10 μl of culture supernatant was pipetted into 50 μl of reaction buffer consisting of 50 mM Tris (pH 7.6), 7.5 mM KCl, 5 mM MgCl_2, 0.96 mM EDTA, 0.05% Nonidet P-40 (Sigma, St. Louis, MO), 5 μg/ml poly (rA) (Pharmacia Biotech, Piscataway, NJ), 1.6 μg/ml oligo(dT)12-18 (Pharmacia Biotech), 2 mM DTT, and 10 μg/ml [α-32P]cAMP (Amersham Life Science Products, Arlington Heights, IL) added immediately before use. This mixture was incubated for 1 h at 37°C, then terminated by the addition of SDS to 0.1%. A sample of reaction mix (5 μl) was spotted onto DEAE filter mats (Wallac, Turku, Finland), air dried for 60 min, and washed 3 times for 10 min in 2× SSC, then twice in 95% ethanol. The filter was dried for 10 min in a vacuum oven at 80°C, before being added to a bag containing 4 ml of beta plate scintillation fluid (Wallace) and counted in a Wallac 1450 beta plate counter. Results were expressed as the mean cpm incorporated in duplicate reactions.

In an additional series of experiments, spleen cells from uninfected 166 transgenic mice were cultured as above at a 1:1 or 1:10 ratio, with a fixed

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number of MT-4 cells/ml in the presence of either M. avium (10⁶ CFU/ml), T. gondii Ts-4 strain tachyzoites (10⁵/ml), or with LPS (100 ng/ml; Sigma) as a positive control. Negative controls consisted of MT-4 cells exposed to the same tachyzoites in the absence of spleen cells. Aliquots of culture supernatants were harvested at 2-day intervals and assayed for RT activity as described above.

Localization of viral expression by in situ hybridization

Portions of spleen, lungs, and liver from M. avium-infected mice were fixed in 1.3 M aqueous formaldehyde for 24 h. In situ hybridization (ISH) was performed as previously described (26). Briefly, two sets of mounted 6-μm paraffin sections were dewaxed and treated with protease to expose viral nucleic acid. They were hybridized with a 3²P-labeled antisense probe (HIV-1, HBB) that represents 9 kb of the HIV-1 genome. A sense probe hybridization was also performed as a control. After hybridization, one set of slides was dipped in NTB 2 Kodak emulsion (undiluted), and the other was used for phosphor storage imaging (27) on a Fuji BAS 5000 instrument.

The autoradiograms were exposed in the dark offi desiccant for 4 days at 4°C and then developed in Kodak D-19 (Eastman Kodak, Rochester, NY) for 4 min at 15°C. The phosphor storage images were analyzed with a MacBas ver.2 software to determine the number of cells expressing HIV RNA per unit area. Alternatively, autoradiograms were stained with hematoxylin and eosin for morphological and pathological assessment. Some slides were stained with Kinyoun’s carbol fuchsin after development and carefully decolorized to preserve the silver grains. This procedure allowed simultaneous detection of bacteria and HIV-expressing cells (15). In other instances, slides were stained with a rat mAb (clone M3/84; PharMingen, San Diego, CA) to Mac-3 to detect cells of the monocyte/macrophage lineage. The Ab was applied using commercial reagents for immunoperoxidase detection (Dako, Carpinteria, CA).

Detection of p24 Ag levels in plasma and ex vivo culture supernatants

At various time points pre- and postinfection with M. avium, transgenic mice were bled from the tail vein into EDTA-treated vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Plasmas were separated and kept frozen at -20°C before assay. p24 antigenemia was determined by ELISA using a commercial kit (Coulter, Miami, FL; detection limit, 4 pg/ml) according to the manufacturer’s instructions. To control for individual variations in basal p24 levels between animals, antigenemia was calculated as the fold increase in p24 vs the preinfection level for each individual mouse. The means and SE were then calculated on the pooled values from all animals assayed at each time point.

In additional experiments, ex vivo production of p24 by freshly isolated spleens from infected and control mice was assessed by incubating single cell suspensions (5 x 10⁶/ml) for 48 h at 37°C in a culture medium consisting of RPMI 1640 (Life Technologies), 10% FCS (HyClone) MEM nonessential amino acid solution (0.1 mM final concentration) (Life Technologies), 5.5 x 10⁻³ M 2-ME (Life Technologies), 10 mM Hepes (Life Technologies), 100 U/ml penicillin (NIH stock), 100 μg/ml streptomycin (NIH stock), and 2 mM glutamine (NIH stock). p24 levels were then determined by ELISA as described above.

In vitro stimulation of p24 production in splenocyte or peritoneal macrophage cultures from noninfected transgenic mice

To assess the effects of T cell vs macrophage stimuli on HIV-1 expression, spleen cells from uninfected line 166 mice were cultured for 72 h at 5 x 10⁶/ml in RPMI 1640 culture media (see above) and stimulated in vitro with immobilized anti-CD3 (10 μg/ml), Con A (Sigma; 2.5 μg/ml), LPS (Sigma; 100 ng/ml), Staphylococcus aureus Cowan (SAC; Calbiochem-Behring, La Jolla, CA; 0.015%), or M. avium (10⁶/ml). Production of p24 in 48-h and IFN-γ in 72-h culture supernatants was measured by two-site ELISA. The IFN-γ assay used immobilized HB 170 mAb, a polyclonal monospecific rabbit anti-mouse IFN-γ Ab, and peroxidase conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as previously described (28). In one set of experiments, peritoneal macrophages obtained from mice 5 days after elicitation with sterile 3% thioglycollate, as described previously (12), were stimulated with M. avium, and the p24 response was measured using the above culture and ELISA protocols.

Phenotypic analysis of HIV-1-expressing cells by fluorescence in situ hybridization and flow cytometry

To assess the cell surface phenotype of virus-producing cells, 2 x 10⁶ spleen cells from M. avium-infected line 166 mice were incubated in FACs buffer (balanced salt solution, 0.1% BSA, 0.01% sodium azide) with FcR block (PharMingen; anti-CD16/32, 10 μg/ml) for 10 min at +4°C and then stained with PE-labeled Ab (PharMingen; anti-CD4, -CD8, -CD19, and -Mac1) for 10 additional min, at +4°C. The cells were then washed twice with FACs buffer and fixed in 100 μl of PermeaFix (Ortho Diagnostics, Raritan, NJ) for at least 1 h at room temperature. Following further washes first in sterile PBS and then in 2X SSC, the cells were pelleted and resuspended in hybridization solution (2X SSC, 30% formamide, sonicated salmon sperm, and yeast transfer RNA) containing 500 ng of 5-carboxy-fluorescein double-end-labeled, gag-pol-specific oligonucleotides probes or gag-pol pool, CA; 0.015%) or M. avium (10⁶/ml). The intracellular hybridization was performed at 42°C for 1 h, followed by successive washes at 42°C in 2X SSC, 0.1% Triton X-100, and in 0.1X SSC, 0.1% Triton X-100. Finally, the cells were resuspended in PBS (pH 8.3), and two color analysis was performed using an Epic XL flow cytometer (Coulter).

Results

Infection with M. avium results in elevated HIV-1 gag transcripts in tissues of transgenic mice

M. avium is a Gram-positive, acid-fast bacterium that is a major cause of opportunistic infection in AIDS patients (30). In common with T. gondii, the microbial stimulus previously studied in this system (12), M. avium is an intracellular pathogen that induces strong proinflammatory and Th1-type cytokine production but differs from that protozoan in selectively infecting macrophage/monocytes (20, 23, 30). To assess the capacity of M. avium to alter HIV-1 expression in vivo, line 166 transgenic mice were infected i.v. with 10⁷ CFU/animal. As a positive control, a second group of transgenic mice was infected with 20 cysts of the ME-49 strain of T. gondii. At 7 and 14 days postinfection, spleens, livers, and lungs were removed from the animals, and cell suspensions were prepared. HIV-1 mRNA expression was then assayed on a fixed number of cells by semiquantitative RT-PCR using primers for the gag gene. As shown in Fig. 1, M. avium induced 2.5- to 5-fold increases in gag mRNA in spleen relative to comparable tissue from noninfected mice. These responses were detected at 7 days and increased further by the second week of infection. Comparable changes in mRNA levels were observed in liver and lungs of the same M. avium-infected animals whereas no viral RNA was detected in infected control (nontransgenic) FVB/N mice at any time, although the recovery of bacteria from these animals was indistinguishable from that measured in transgenic mice (data not shown). Confirming our previous work utilizing Northern analyses of viral mRNA production (12), increased gag expression was also detected by RT-PCR in spleens of mice infected with T. gondii. (Fig. 1). The above data establish that mycobacterial as well as protozoan infections can stimulate HIV-1 mRNA expression in tissues of line 166 transgenic mice.

Infection of transgenic mice with M. avium or T. gondii results in enhanced production of infectious virions

Since transgenic mice carrying full-length copies of proviral DNA possess the biosynthetic machinery necessary for the assembly of HIV-1 virions (17), we asked whether the elevations in viral mRNA seen in tissues of infected line 166 mice would be reflected in the induction of infectious HIV-1. To assess this possibility, spleen cells from M. avium- or T. gondii-infected transgenic mice were cocultured with the human T cell line MT-4 at a ratio of 1:1, 1:10, and 1:100, and supernatants were assayed at 2-day intervals for the presence of RT. Because RT activity is nearly exclusively virion associated, its amplification in T cell cultures is a useful

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yields observed in the spleens of line 166 mice infected with M. avium infections. Each cDNA was titrated to confirm that the reaction was optimal and results were normalized against PCR product for HPRT to ensure equivalent amounts of cDNA in each reaction. The figure shows typical results from a representative mouse (cDNA at 5 ng/ml). The graphs presented show the geometric mean and SE of samples derived from five mice, comparing the fold increase in expression at days 7 and 14 postinfection with that of uninfected animals.

indicator of spreading infection by viral particles. Supernatants from cocultures containing spleen cells from uninfected line 166 mice displayed measurable although low levels of RT, confirming that the proviral transgenes in these animals can indeed be expressed and that their products yield infectious HIV (Fig. 2A). This RT activity was detected only at the highest concentration of splenocytes employed, suggesting that the number of cells releasing virus in uninfected animals is quite small. In contrast, higher levels of RT were produced in cultures containing the same number of spleen cells from mice infected with either M. avium or T. gondii, and this response was retained at a 1:10 dilution of the splenocytes relative to the MT-4 indicator cells whereas all activity was lost upon an additional 10-fold dilution (Fig. 2, B and C). These results suggest that the initial spleen cell populations from the M. avium- and T. gondii-infected mice produced infectious virions at a level at least 10 times that generated by splenocytes from uninfected mice.

To be certain that the RT activity observed was HIV-1 specific, MT-4 coculture experiments were conducted under identical conditions using spleen cells derived from M. avium- or T. gondii-infected nontransgenic FVB/N control animals. Significant RT activity was never observed under these conditions. Similarly, culture of spleen cells from infected line 166 mice in the absence of MT-4 cells also repeatedly failed to produce detectable RT (data not shown), confirming that the murine cells themselves cannot be the major source of the activity measured. Together, the above data indicate that the elevations in HIV-1 mRNA induced by infection in line 166 mice reflect increased titers of productively assembled infectious virus.

Nonetheless, there remained the possibility that the greater viral yields observed in the spleens of line 166 mice infected with M. avium or T. gondii reflect the recruitment of preexisting HIV-1-producing cells into that organ rather than the de novo induction of virus from normally silent splenocytes. To confirm that these microbial agents are capable of directly stimulating virus production, spleen cells from uninfected 166 transgenic mice were cultured at a 1:1 or 1:10 ratio with a fixed number of MT-4 cells per milliliter in the presence of either M. avium (10^6 CFU/ml), T. gondii tachyzoites of the avirulent Ts-4 strain (10^6/ml), or with LPS (10 μg/ml) as a positive control. As shown in Fig. 3, in vitro exposure of transgenic spleen cells to each of these stimuli (Fig. 3, B–D) respectively resulted in a 10-fold or better increase in the yield of RT activity relative to that displayed by nonstimulated control cultures based on cell dilution (Fig. 3A). Addition of any of these microbial agents to MT-4 cells in the absence of transgenic spleen cells, or to transgenic T cells in the absence of MT-4 cells, failed to induce detectable RT activity (data not shown). Together, the above in vitro findings argue that up-regulated HIV-1 expression observed in vivo upon T. gondii or M. avium infection can result at least in part from the de novo induction of viral synthesis in mouse cells.

Infection with M. avium results in systemic elevation in viral protein

To further confirm that the increased viral loads seen in tissues of pathogen-infected transgenic animals are due to the induction of HIV-1 expression rather than solely to cell migration, we measured levels of the p24 core protein in plasma as a parameter of systemic viral production. M. avium was used as the infecting agent in this
and all subsequent experiments because of the ease of quantitating the infection and for the purposes of standardizing a defined model for future studies. Low and variable amounts (100–400 pg/ml) of p24 protein were detectable by ELISA in plasma of uninfected 166 mice. However, in animals infected with *M. avium* (10⁸ CFU/mouse, administered i.v.), plasma p24 levels rapidly increased to 3-fold the preinfection values at wk 1 and then gradually declined to baseline by wk 5–6 postinfection. In contrast, in plasma from age-matched uninfected transgenic mice, p24 levels remained stable during the same period with only minor fluctuations (Fig. 4A).

To assess whether the increased levels of p24 in the plasma of *M. avium*-infected transgenic mice directly reflect augmented production of the viral protein in tissues, spleen cells from *M. avium*-infected or uninfected transgenic control mice, taken at different time points postbacterial inoculation, were cultured for 48 h, and the supernatants were assayed for p24 by ELISA. Splenocytes from *M. avium*-infected mice secreted significantly more p24 than cells from uninfected controls, starting at wk 1 postinfection with a greater than 10-fold increase and rising to over 100-fold by wk 4 and 6 (Fig. 4B). This change roughly paralleled the increase in bacterial loads (from 0.53 × 10⁸ at wk 1 to 22.3 × 10⁸ CFU/spleen at wk 6) measured in the same cells during the same period (Fig. 4B). Nevertheless, the time course of p24 expression in spleen was almost the converse of that in the plasma (Fig. 4A), suggesting that these two read-outs reflect distinct events associated with virus production in vivo.

**Localization of *M. avium*-induced viral expression in tissues**

The preceding experiments demonstrated that pathogens such as *T. gondii* or *M. avium* can induce the expression of both HIV-1 mRNA and infectious virus from transgenic murine cells. Since, in a transgenic animal presumably every cell contains proviral DNA and is therefore in principle capable of viral synthesis and secretion, an important question concerns whether the HIV-1 induced by microbial immune activation originates from specific cell types. As a first step in examining the cellular origins of the induced virus, tissues from *M. avium*-infected line 166 mice were embedded in paraffin, sectioned, and protease-treated to expose nucleic acids, and ISH was performed with labeled antisense riboprobe derived from the HIV genome. No detectable hybridization was observed with tissues of nontransgenic littermates (data not shown) or with tissues of *M. avium*-infected transgenic mice with a control sense riboprobe (e.g., Fig. 5A). Very low levels of hybridization (antisense) were detected in all tissues sampled from uninfected line 166 mice, the signals appearing sporadically and usually small in size (Figs. 5B and 6A). However, when examined at day 14 postinfection, the hybridization signals had clearly increased in both frequency and intensity in spleen (Fig. 5C), lung (Fig. 6B), and liver (not shown). Densitometric quantitation revealed an approximate 4- to 5-fold increase in hybridization signal.
in tissues of infected vs noninfected animals, consistent with the results of RT-PCR performed on similar tissues (Fig. 1).

Although precise identification of virus-producing cells was difficult at the level of resolution of the ISH assay, specific patterns of expression in tissues were nevertheless apparent. In spleen, hybridization was largely restricted to the red pulp and infrequently observed in T cell areas (Fig. 5C). Similarly, in the lungs of infected mice, hybridization foci were most abundant within the macrophage-enriched granulomatous lesions resulting from the immune response to M. avium bacilli (Fig. 6, B and D). Colocalization of HIV-1 by ISH and of M. avium by acid fast staining revealed that both bacteria-infected and uninfected macrophages express virus. Indeed, many of the ISH-positive cells, although concentrated largely within the inflammatory lesions, lacked visible bacteria (Fig. 6B). Immunocytochemistry performed on nearby sections confirmed that the majority of cells in the areas of inflammation where elevated viral expression was observed were mononuclear phagocytes, as judged by their staining with Ab specific for the Mac-3 surface Ag marker (Fig. 6D).

Ex vivo analysis of HIV-1-expressing cell population from M. avium-infected mice

To confirm that macrophages constitute the major population producing virus in response to M. avium infection, spleen cells from 7-day-infected line 166 mice were stained with PE-labeled mAb against different lymphocytic and mononuclear cell surface markers, and FISH was performed with fluoresceinated oligonucleotides specific for HIV gag-pol RNA. Dual color FACS analysis was then performed on the stained populations. As shown in Table I and Fig. 5, in spleens of mice infected with M. avium, the percentage of Mac-1+ cells expressing HIV gag-pol RNA was dramatically increased, as compared with splenocytes from uninfected animals. This approximate 12-fold augmentation in HIV mRNA-expressing cells (from 0.4 to 4.9%) could not be explained by the increased percentage of Mac-1+ cells in the spleen (2.4% in uninfected mice vs 5.4% in M. avium-infected mice). In contrast, no substantial elevations were observed in the percentage of HIV gag-pol RNA-expressing cells within the populations stained with T or B cell markers (CD4+, CD8+, or CD19+ cells) (Table I). These experiments argue that macrophages rather than lymphocytes are the major source of HIV-1 expression in spleens of infected transgenic mice.

Analysis of virus production in in vitro-stimulated spleen cell cultures

One possible explanation of the finding that M. avium infection preferentially induces virus production from macrophages in transgenic mice is that the former pathogen preferentially invades and stimulates these cells. To ascertain whether the observed macrophage responses are not pathogen specific, a series of in vitro experiments were performed in which splenocytes from uninfected line 166 mice were exposed in vitro to known monocyte and/or T cell stimuli. As shown in Table II, immobilized anti-CD3 or Con A failed to induce significant increases in p24 production compared with control cultures. In contrast, LPS and SAC stimulated greater than 20-fold increases in p24 levels. The failure of the T cell stimuli to induce viral expression is not due to a lack of activation of the lymphocytes since anti-CD3 and Con A triggered strong IFN-γ responses in the same cultures. In agreement with both the in vivo studies described above (Figs. 1, 2, 4, and 5; Table I) as well as the results of the in vitro experiments measuring expression of HIV-1 by MT-4 culture assay (Fig. 3), M. avium was found to directly stimulate p24 production by spleen cells from uninfected transgenic mice (Table II) and induced a highly significant p24 response in enriched thioglycollate-elicited macrophages from the same animals (345 ± 15 pg/ml in M. avium-stimulated vs 46 ± 15 pg/ml in unstimulated cultures). Together these results suggest that macrophages provide a more readily induced reservoir for HIV-1 expression than T lymphocytes and that M. avium, in common with other microbial agents such as T. gondii, SAC, and LPS, can provide an excellent stimulus for virus production from that cellular source.

Discussion

The data reported in the present study demonstrate that HIV-1 transgenic mice infected with M. avium, a common opportunistic pathogen encountered by AIDS patients, display increased viral mRNA levels in tissues as previously reported for transgenic animals exposed to T. gondii (12). More importantly, we now establish that this increase in HIV-1 gene expression within infected organs is reflected in the enhanced production of p24 protein, as
well as infectious virus particles. Finally, we demonstrate that *M. avium*-induced HIV-1 synthesis is localized to specific sites within tissues and appears to be confined both in vivo and in vitro to cells of the macrophage/monocyte lineage. The transgenic mouse/infection model we describe thus may provide a useful tool for studying activation of HIV-1 from its macrophage reservoir and, in particular, the immunoregulation of this response.

A key issue we have addressed concerns the basis of the increased viral expression observed in tissues of *M. avium*- or *T. gondii*-infected mice. These elevations, which ranged between approximately 4- and 10-fold depending on the assay, could reflect either de novo HIV-1 induction and/or the recruitment and localized enrichment of virus-producing cells already present at low levels in transgenic animals before microbial infection. The former explanation is supported by several observations and, most importantly, the systemic increases in p24 in plasma detected during the first few weeks following *M. avium* infection (Fig. 4). Moreover, focal in situ hybridization signals appeared to be more intense in tissues of infected transgenic mice (Figs. 5 and 6), suggesting augmented transcription from individual cells. Finally, significant increases in infectious virus, as well as released p24, could be stimulated in spleen cells in vitro (Fig. 3), a finding that is most consistent with the direct induction of viral expression. Nevertheless, a partial contribution of tissue-specific cell recruitment, and in particular of macrophages (Table I), cannot be ruled out and in fact is predictable, given the mononuclear inflammatory responses observed in the tissues studied. Such a recruitment process, if occurring in HIV-1 humans, could play a role in promoting viral spread.

**Table I.** Characterization of HIV-1-expressing cell populations in spleens of *M. avium*-infected line 166 mice by combined FISH and FACS

| Mouse Group       | Cell Surface Stain | % of Total Spleen | % FISH-Positive Cells
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>Anti-Mac-1</td>
<td>2.4</td>
<td>0.4</td>
</tr>
<tr>
<td><em>M. avium</em>-infected</td>
<td>Anti-Mac-1</td>
<td>5.3</td>
<td>4.9</td>
</tr>
<tr>
<td>Uninfected</td>
<td>Anti-CD4⁺ 8</td>
<td>39.5</td>
<td>≤0.2</td>
</tr>
<tr>
<td><em>M. avium</em>-infected</td>
<td>Anti-CD4⁺ 8</td>
<td>46.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Uninfected</td>
<td>Anti-CD19</td>
<td>22.3</td>
<td>≥0.2</td>
</tr>
<tr>
<td><em>M. avium</em>-infected</td>
<td>Anti-CD19</td>
<td>15.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*a* Pooled spleen cells from three uninfected or *M. avium*-infected (7-day) line 166 mice were stained with PE-labeled Ab (anti-CD4, -CD8, -CD19, or -Mac-1) and fixed at room temperature. After washing, intracellular hybridization was performed at 42°C with 500 ng of 5-carboxyfluorescein double-end-labeled, gag-pol-specific oligonucleotide probes or gag-pol sense oligonucleotides as a negative control probe. The cells were then resuspended in PBS, and FACS was performed on a flow cytometry (Epic XL; Coulter). No significant fluorescein staining was obtained with the sense control probe in any of the samples or when cells from nontransgenic mice were substituted for transgenic cells in the assays (FISH + cells ≤ 0.2%).

*b* Values obtained by gating on cell surface expression.
The explanation for this differential response pattern in plasma and spleen is presently unclear. One possibility is that it reflects changes in cytokine expression occurring during the same period in *M. avium*-infected mice. For example, while IFN-γ synthesis is induced early and reaches a plateau at 2 wk (20, 23), IL-10 production is delayed and does not occur at high levels until 3–4 wk postinfection (D. Jankovic and A. Sher, unpublished observations). The decline in plasma p24 levels may represent a response to this burst in IL-10 synthesis. Preliminary data (not shown) indicate that p24 synthesis by spleen cells also becomes diminished at later time points (i.e., >6 wk), perhaps as a consequence of the same IL-10 regulatory response. Experiments in which IL-10 has been experimentally depleted or administered during the course of *M. avium* infection in transgenic mice are in progress to assess the possible role of this down-regulatory cytokine in the control of immune activation-induced viral expression.

Although the HIV-1 proviral transgene should be present in all somatic cells in line 166 mice, viral expression in response to *M. avium* appears to arise from a restricted cellular source. This conclusion is evident, from the anatomical localization of HIV-1 expression in situ, from the preferential expression of viral mRNA ex vivo by Mac-1⁺ cells from infected mice, and from the induction of virus expression in vitro by LPS or SAC but not Con A or anti-CD3. Taken together with our previous observations in *T. gondii*-infected transgenic mice indicating that viral mRNA is produced preferentially by adherent cells in spleen and can be stimulated in thioglycollate-elicited peritoneal macrophages (12), these findings strongly implicate cells of the macrophage/monocyte lineage as the principal source of HIV-1 expression in line 166 mice infected with either pathogen and argue against a T or B lymphocyte origin. Although we cannot rule out a role for dendritic cells in virus production, the paucity of HIV-1⁺ cells in the white pulp of spleens from *M. avium*-infected mice (Fig. 5A) suggests that interdigitating dendritic cells are not involved.

Despite strong microbial stimulation in vivo or polyclonal stimulation in vitro, T lymphocytes from transgenic mice failed to exhibit elevated viral expression although integrated proviral DNA was clearly present in FACS-sorted cells of this lineage (data not shown). Since the HIV-1 molecular clone used to construct the mouse was T tropic, the unresponsiveness of the transgenic T cells appears to be unrelated to the cellular tropism of that virus. Instead, the data suggest that virus-infected cells of the macrophage/monocyte lineage may be intrinsically more susceptible than T lymphocytes to immune activation. This distinction may be less apparent in persistently infected HIV-1⁺ human cells because of differences in initial infection levels between T cells and macrophages and because of ongoing receptor-mediated viral entry.

The above considerations suggest that the *M. avium*-infected transgenic mouse model we have described offers a powerful experimental tool for studying the immune activation of integrated HIV-1 from its in vivo macrophage reservoirs. Although present in lower numbers than HIV⁺CD4⁺ T lymphocytes (31, 32), macrophages containing HIV-1-integrated DNA can constitute a major source of persistent infection since they are relatively resistant to viral cytopathic effects. Macrophages with HIV DNA have been isolated from lymph nodes and spleens throughout the course of infection, while macrophages expressing HIV mRNA are more difficult to detect, suggesting that they may serve as a protected site for replication-competent provirus (33). In addition, in certain anatomical sites, such as the central nervous system, monocyte/macrophages are the only infected cells detected in situ (34–35). Little information exists on the survival of latently infected macrophages after potent antiretroviral therapy (36), although HIV within macrophages has been reported to be relatively resistant to protease

FIGURE 7. FACS dot-plot profiles of HIV gag-pol-expressing cells. Splenocytes from *M. avium*-infected (top panel) and uninfected (lower panel) line 166 mice were analyzed for the in situ expression of HIV gag-pol mRNA by FISH (FITC staining) and for the surface expression of Mac-1 (PE staining). Expression was analyzed in an enriched Mac-1⁺ population by gating on large cells on forward scatter. The profiles shown are from the experiment presented in Table I.

Since *M. avium*, in common with both *T. gondii* and *M. tuberculosis*, stimulates strong proinflammatory and Th1-type host cytokine responses, its ability to trigger viral expression in transgenic mice was not unexpected and confirms previous reports describing an association of this bacterial infection with HIV-1 expression in tissues of AIDS patients (15, 16). Importantly, the transgenic mouse model has enabled us to follow for the first time the kinetics of viral expression following *M. avium* infection. This analysis revealed a rapid increase in p24 levels in both spleen cell supernatants and plasma by the first week postinfection. Interestingly, while splenic p24 production continued to rise during the next 5 wk consistent with the growing bacterial load in that organ, plasma p24 levels declined in an approximate reciprocal fashion (Fig. 4).
inhibitors in vitro (37). Given that integrated HIV-1 DNA within resting CD4+ T cells can persist in patients receiving prolonged highly active antiretroviral therapy (31, 38, 39), it is likely that virus-infected macrophages are also refractory.

Monocytes/macrophages may be of particular importance as reservoirs for microbiologically stimulated virus production (1, 11, 15, 16), and thus the ability to infect mice with bacterial and parasitic pathogens commonly found in HIV-1 individuals offers a unique advantage for studying this interaction in vivo. Because of the rapid (1–2 wk) response of line 166 to microbial (M. avium or T. gondii) infection and the ease of measuring this response by p24 antigenemia in plasma or cell culture supernatants, the model allows the rapid testing of interventions that inhibit or augment immune activation and could be utilized to assess the susceptibility of the macrophage reservoir to antiretroviral or immune-based therapies. We are currently pursuing these approaches with the aim of both better understanding the requirements for microbially induced viral expression and of developing strategies for limiting the impact of that process on HIV-1 progression.

Acknowledgments

We thank Malcolm Martin, for supporting the transgenic mouse breeding facility used in this project, and Jay Berzofsky, Ricardo Gazzinelli, and Sharon Wahl, for their helpful advice and criticism.

References


carcinosis factor a activates human immunodeficiency virus type 1 through induc-

tion of nuclear factor binding to the NF-kB sites in the long terminal repeat. Proc.

Natl. Acad. Sci. USA 86:5974.


Table II. In vitro p24 production by spleen cells from uninfected transgenic mice is preferentially up-regulated by macrophage stimuli

<table>
<thead>
<tr>
<th>Experiment</th>
<th>In Vitro Stimulus</th>
<th>p24 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>179.2 ± 70.96</td>
<td>960 ± 174</td>
</tr>
<tr>
<td></td>
<td>Immobilized anti-CD3</td>
<td>319.3 ± 103.0</td>
<td>22,373 ± 3,770</td>
</tr>
<tr>
<td></td>
<td>Con A</td>
<td>114.2 ± 26.7</td>
<td>25,751 ± 7,308</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>382.4 ± 1,453.5</td>
<td>1021 ± 135</td>
</tr>
<tr>
<td></td>
<td>SAC</td>
<td>3949.1 ± 1204.4</td>
<td>17,082 ± 2,965</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>28.9 ± 6.4</td>
<td>&lt;30</td>
</tr>
<tr>
<td></td>
<td>M. avium</td>
<td>253.1 ± 151.0</td>
<td>241.8 ± 38.1</td>
</tr>
</tbody>
</table>

* Spleen cells were cultured at 5 × 10⁷/ml in the presence or absence of immobilized anti-CD3 (10 μg/ml), Con A (2.5 μg/ml), LPS (100 ng/ml), SAC (S. aureus Cowan, 0.01%), or M. avium. Production of p24 and IFN-γ in supernatants was measured 48 or 72 h later by two site ELISA.

* Mean ± SE of ELISA readings on spleen cells from four individual mice.

* Mean ± SE of ELISA readings on pooled spleen cells from three mice.