Blocking of retroviral infection at a step prior to reverse transcription in cells transformed to constitutively express interferon β

(defective Moloney murine leukemia virus/human immunodeficiency virus/antiviral gene therapy)

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ABSTRACT We are developing methods for somatic-cell gene therapy directed against infection with human immunodeficiency virus, by enhancing antiviral resistance of target cells through the constitutive production of autocrine interferon (IFN). Using the human IFN- β coding sequence under the constitutive low-expression control of a 0.6-kb murine *H-2K^b* promoter fragment, we have constructed a retroviral vector, HMB-K^bHuIFN β , and have transformed cells of the T98G human neuroblastoma line, the U-937 human promonocytic line, and the CEM human lymphocytic line. These human IFN- β -transformed cell populations have acquired a low, constitutive production of human IFN, while replicating at a rate similar to that of untransformed cells and of cells transformed with the control vector carrying a human IFN- β sequence encoding an inactive, mutated protein. In the three different cell populations tested, transformation with the HMB-K^bHuIFN β vector resulted in a 1.3–2.3 log₁₀ reduction in the number of cells infected with a defective amphotropic MFG-LacZ retrovirus. A kinetic study of the fate of the MFG-LacZ retrovirus in the culture medium and intracellularly immediately after exposure of the cells to virus revealed a significant reduction of the appearance of intracellular virus in human IFN- β -transformed cells. A similar effect was obtained by treating untransformed T98G, U-937, and CEM cells with exogenous human IFN- β . The blocking effect of autocrine or exogenous human IFN- β on viral entry was not limited to virus specific for the amphotropic receptor but was also obtained in murine IFN- β -treated NIH 3T3 mouse fibroblasts infected with an ecotropic MFG-LacZ retrovirus. Infection of human IFN- β -transformed CEM cells with human immunodeficiency virus type 1 gave comparable results. Immediately following exposure of the cells to human immunodeficiency virus, a kinetic study of the fate of the virus failed to reveal the appearance of intracellular virus and showed that the majority of the input virus remained in the extracellular medium. We conclude that low autocrine IFN- β synthesis, or exposure of cells to exogenous IFN- β , prevents virus from getting inside the cells, regardless of the virus receptor involved.

The broad-spectrum antiviral activity of interferons α and β (IFN- α and - β) results from the action of IFN-induced proteins (1). The antiviral state has been shown to interfere with retroviral replication, and, until recently, it was believed that IFNs disrupted late stages of the infectious cycle, enhanced the formation of noninfectious virions, and blocked the release of viral particles from the cell surface membrane (2). Effects on earlier stages have been observed with Rous sarcoma and murine sarcoma virus, suggesting that an IFN-induced block can act at some early step of infection and result in decreased integration of proviral DNA (3, 4). Using

an infectious, replication-incompetent, Moloney murine leukemia virus, Kumar and Sen (5) have come to the conclusion that murine IFN- β treatment of murine cells results in the inhibition of infection at the stage of retroviral integration or perhaps at a stage prior to it. One of these steps could be the inhibition of uncoating of retroviral particles, as suggested by Aboud *et al.* (6). The AIDS epidemic has restimulated an interest in the effects of IFNs on retroviral replication, and it has become evident that stages of the retroviral cycle prior to assembly and release can be affected by IFNs. In a study of the action of IFN on human immunodeficiency virus (HIV) infection of primary macrophages, Kornbluth *et al.* (7) report a decreased formation of proviral DNA under the influence of IFN, whereas Shirazi and Pitha (8) report that IFN- α treatment of lymphocytic CEM cells inhibits the first replication cycle of HIV-1 in these cells and decreases the levels of viral RNA and proteins. Those authors suggest that IFN- α activity could be at the level of either the formation or the integration of proviral DNA, a conclusion also supported by the work of Meylan *et al.* (9) on primary macrophages infected with HIV-1.

We are exploring ways of using IFN genes for somatic-cell gene therapy directed against HIV and are developing methods for obtaining stable antiviral expression (SAVE) (10) in cells by introducing an IFN gene placed under constitutive low-expression control compatible with cell survival and proliferation. Previous work achieved SAVE in clones of murine BALB/c 3T3 cells and human promonocytic U-937 cells transformed by plasmids carrying the murine and the human IFN- β coding sequence placed under the transcriptional control of a 0.6-kb murine *H-2K^b* gene promoter fragment (10, 11). We have now constructed retroviral vectors carrying the human IFN- β genomic sequence under the low-expression control of the 0.6-kb fragment of the *H-2K^b* promoter and have transformed three human cell lines, T98G, U-937, and CEM. Such human IFN- β -transformed cells have become resistant to infection with an amphotropic retrovirus and with HIV-1 at the very early stage of viral entry, at the level of either adsorption or penetration.

MATERIALS AND METHODS

Cell Lines, IFNs, and Viruses. The ecotropic ψ -CRE cell line and amphotropic ψ -CRIP cell line (12) and the NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. The human T98G glioblastoma tumor cells and the human WISH amnion cells were cultivated in DMEM

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Abbreviations: HIV, human immunodeficiency virus; SAVE, stable antiviral expression; IFN, interferon; RT, reverse transcriptase; VSV, vesicular stomatitis virus; SFV, Semliki Forest virus; cfu, colony-forming unit(s).

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enriched with 10% fetal bovine serum. The human U-937 promonocytic line and the human CEM subclone (CEM13) were cultivated in RPMI 1640 medium containing 10% fetal bovine serum. Recombinant human IFN- β was provided by Bioferon (Laupheim, Germany). Murine IFN- α/β was prepared in C-243 cells (10). Vesicular stomatitis virus (VSV; Indiana strain) and Semliki Forest virus (SFV; Kumba strain) stock suspensions had been prepared in chicken embryo fibroblast cultures (10). The HIV-1_{BRU} stock was prepared in THP-1 cells [reverse transcriptase (RT) activity close to 6×10^6 cpm/ml] (13).

Packaging Clones. The pHMB-K^bHuIFN β , pHMB-K^bHuIFN β delStyI, or pMFG-LacZ (14) vectors were introduced into ψ -CRIP cells by electroporation (15). Several packaging clones were obtained: ψ -CRE-MFG-LacZ at a titer of 2×10^6 colony-forming units (cfu)/ml, ψ -CRIP-MFG-LacZ at 5×10^5 cfu/ml, ψ -CRIP-HMB-K^bHuIFN β delStyI at 10^4 cfu/ml, and ψ -CRIP-HMB-K^bHuIFN β at 10^5 cfu/ml. The absence of helper-virus production by the packaging clones was confirmed by using a sensitive marker-rescue assay. Viral supernatants were concentrated by polyethylene glycol (PEG) treatment (16).

Transformation of Human Cells by the HMB Retroviral Vectors. T98G, U-937, and CEM cells were infected with viral supernatants containing HMB-K^bHuIFN β or HMB-K^bHuIFN β delStyI particles at a multiplicity of infection close to 1. Three weeks after transformation, cells were analyzed for retroviral integration, human IFN production (11), and antiviral resistance. Flow cytometry analysis of CD4 antigen in the various CEM cell populations was performed as described (11).

Antiviral Resistance. The antiviral resistance of HMB-transformed cells against VSV or SFV was assessed as described (11). Resistance against infection with the replication-defective MFG-LacZ retrovirus was determined by infecting exponentially growing cells with serial 10-fold dilutions (10^{-2} to 10 cfu/cell) of a MFG-LacZ supernatant. Two days after infection, cells were stained for expression of β -galactosidase activity (15).

RT Activity in Culture Supernatants and Cell Extracts. Fifty thousand cells (T98G, U-937, CEM, NIH 3T3) were infected in 1 ml of culture medium with MFG-LacZ retroviruses, and 5×10^4 CEM cells were infected in 0.5 ml of culture medium with HIV. RT activity was assayed (17) in triplicate in cell extracts and culture media at various time intervals after infection. Cell extracts were prepared after the cells were harvested and washed three times with cold phosphate-buffered isotonic saline. Cells infected by MFG-LacZ retrovirus were suspended in 50 μ l of hypotonic buffer (10 mM Tris, pH 7.5/10 mM NaCl/1.5 mM MgCl₂) and disrupted by sonication (50 kHz, 30 sec). Cells infected by HIV-1 were disrupted by incubation at 4°C for 10 min in 50 μ l of the same hypotonic buffer supplemented with 1% (vol/vol) Nonidet NP-40 and cellular extracts were centrifuged at $13,000 \times g$ for 5 min at 4°C.

RESULTS

Transformation of Human Cells by HMB-Based Vectors. We constructed a retroviral vector plasmid carrying the

human genomic IFN- β coding sequence under control of a 0.6-kb fragment of the murine *H-2K^b* promoter (pHMB-K^bHuIFN β) (Fig. 1). A control construction expressing a truncated, inactive human IFN- β protein, the pHMB-K^bHuIFN β delStyI vector, was also prepared. T98G, CEM, and U-937 human cell lines were transformed with supernatants of ψ -CRIP-HMB-K^bHuIFN β and ψ -CRIP-HMB-K^bHuIFN β delStyI packaging cells at 1–3 copies per cell.

Production of human IFN. No functional human IFN was detected in the supernatants of control cell populations (untransformed and transformed by HMB-K^bHuIFN β delStyI), whereas cells transformed by HMB-K^bHuIFN β secreted human IFN ranging from 5 to 9 units per day per 5×10^4 cells.

Induction of 46-kDa (2'–5')oligoadenylate synthetase and 68-kDa protein kinase in HMB-transformed T98G cells. The presence of transcripts for the 46-kDa (p46) (2'–5')oligoadenylate synthetase and the p68 protein kinase, two key enzymes of the IFN-induced antiviral response, was studied in T98G cells by PCR amplification. This showed that (2'–5')oligoadenylate synthetase and p68 kinase transcripts were, respectively, 15- and 3-fold more abundant in human IFN- β -transformed cells than in control cell populations (Fig. 2).

Replication of vector-transformed cells. Cells were kept in culture for at least 3 months with two passages per week. For all three cell lines, T98G, CEM, and U-937, we observed no difference in replication rate between control cells and human IFN- β -transformed cells, indicating that the constitutive expression of human IFN- β was low enough to be compatible with normal cell replication.

Constitutive Antiviral State of Human IFN- β -Transformed Cells. Cytolytic viruses. Human IFN- β -transformed cells displayed various degrees of resistance against VSV and SFV, ranging from 0.8 to 2 log₁₀ units (Table 1).

Amphotropic defective MFG-LacZ retrovirus. A significant reduction in the number of β -galactosidase-expressing cells, ranging from 1.3 to 2.3 log₁₀ resistance units, was observed in the human IFN- β -transformed cell populations (Table 1). We therefore defined the stages at which the inhibition of retrovirus infection occurred.

The presence of the MFG-LacZ provirus in the genomic DNA of HMB-transformed T98G, CEM, and U-937 cells 48 hr after infection with 1 cfu per cell was studied by Southern blot analysis. Whatever the cell type analyzed, the constitutive expression of human IFN- β (Fig. 3, lanes 6, 14, and 20) resulted in at least 10-fold reduction of the levels of the 5-kb fragment of the MFG-LacZ proviral DNA. Southern blot analysis of Hirt supernatant (19) extracted 10 hr after infection showed that unintegrated viral DNA was undetectable in IFN- β -transformed T98G cells (data not shown), demonstrating that the block occurred either at the stage of viral DNA synthesis or prior to it.

Therefore, it seemed logical to examine earlier steps of the infectious cycle, and a series of experiments was set up in which the fate of viral particles was followed by measuring viral RT activity in the medium and in the cells after onset of infection with the amphotropic MFG-LacZ vector at 1 cfu per cell. In the culture medium of the control cells, a gradual decrease in RT activity was observed, with only about 10%

FIG. 1. Retroviral vectors. The enhancer-deleted pHMB-neo plasmid (18) was digested with *EcoRI* and *BamHI*, excising the Tn5 *neo* coding sequence under the control of the phosphoglycerate kinase promoter. The *BamHI*–*HindIII* fragment of the plasmid pK^bneoHuIFN β (11) containing the genomic human IFN- β coding sequence and its polyadenylation site (HuIFN) driven by the 0.6-kb *H-2K^b* promoter fragment (*H-2K prom*) was blunt-ended by Klenow DNA polymerase and inserted into the pHMB vector, generating the pHMB-K^bHuIFN β retroviral vector plasmid. The pHMB-K^bHuIFN β delStyI vector, coding for a mutated, inactive human IFN- β , has the same structure as pHMB-K^bHuIFN β except for a 4-bp insertion in the *StyI* restriction site of the human IFN- β coding region. dLTR; the 3' long terminal repeat of Moloney murine leukemia virus with a 327-bp deletion of the enhancer and promoter region.

FIG. 2. Detection of RNA species in HMB-transformed T98G cells by PCR amplification of cDNA. One-sixteenth of the cDNA product (first-strand synthesis kit, Pharmacia) made from 100 ng of total RNA (RNA isolation kit, Stratagene) was amplified to detect the human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcript (982 bp), which serves as a quantitative control in this experiment, a DNA fragment of the expected size (314 bp) corresponding to a human (2-5)oligoadenylate synthetase (OAS) transcript, and a DNA fragment (527 bp) corresponding to the human double-stranded-RNA-activated protein kinase (p68) transcript. The PCR amplification products were analyzed by Southern hybridization using as probe a 32 P-labeled DNA fragment of the human G3PDH cDNA, the human OAS cDNA, and the human p68 cDNA (11). Lanes: C, untransformed T98G cells; Δ IFN; HMB-K^bHuIFN β delStyI-transformed T98G cells; IFN; HMB-K^bHuIFN β -transformed T98G cells.

remaining 6 hr after onset of infection (Fig. 4). Concurrently, the intracellular level of RT activity increased and reached a maximum at 4–6 hr. In contrast, 6 hr after onset of infection, 75%, 48%, and 42% of the RT activity was still present in the culture medium of human IFN- β -transformed T98G, CEM, and U-937 cells, respectively, whereas the intracellular level of RT activity in these cells remained low, close to background. That the RT activity remained at a high level in the

Table 1. Resistance of HMB-transformed human cell populations to VSV, SFV, and amphotropic MFG-LacZ vector

Cell line*	Resistance		
	VSV†	SFV†	MFG-LacZ‡
T98G Δ IFN	0	ND	0.1 $\pm$ 0.1
T98G IFN	2.0 $\pm$ 0	ND	2.3 $\pm$ 0.1
CEM Δ IFN	0	0	0.1 $\pm$ 0.1
CEM IFN	1.7 $\pm$ 0.6	1.0 $\pm$ 0	1.4 $\pm$ 0.1
U-937 Δ IFN	0	0	0.1 $\pm$ 0.1
U-937 IFN	0.8 $\pm$ 0.3	0.7 $\pm$ 0.3	1.3 $\pm$ 0.2

* Δ IFN, HMB-K^bHuIFN β delStyI-transformed cells, IFN, HMB-K^bHuIFN β -transformed cells.

†Resistance to VSV and SFV is expressed as the log₁₀ value of the difference between the virus titers determined in untransformed and in transformed cells (mean $\pm$ SD of triplicates). ND, not determined.

‡Resistance to MFG-LacZ is expressed as the log₁₀ value of the difference between the retroviral titers determined in untransformed cells and in transformed cells (mean $\pm$ SD of duplicates).

culture medium of human IFN- β -expressing cells reflects an inhibition of the appearance of intracellular virus. The blocking of MFG-LacZ entry into cells was also observed with human cells exposed to exogenous human IFN- β at 2000 units/ml. Similar results were obtained with NIH 3T3 mouse fibroblasts pretreated with murine IFN- α/β at 2000 units/ml and infected with an ecotropic MFG-LacZ retrovirus (data not shown).

Single round of HIV infection. We examined the anti-HIV resistance of human IFN- β -transformed CEM cells. It should be emphasized that the expression of CD4 at the cell surface of the three different CEM populations was identical (data not shown). Transformed and untransformed CEM cells were infected with HIV-1. Two days later, the presence of HIV-1 proviral DNA was significantly lower in the human IFN- β -

FIG. 3. Detection of the MFG-LacZ proviral DNA by Southern blot analysis. The three cell lines (T98G, CEM, and U-937), untransformed or transformed by HMB-K^bHuIFN β or by HMB-K^bHuIFN β delStyI vectors, were infected by the defective amphotropic MFG-LacZ retrovirus at a multiplicity of infection of $\approx$ 1. Genomic DNA was extracted 48 hr later, digested with *Nhe*I restriction endonuclease, and analyzed by Southern hybridization using as probe the 32 P-labeled *Bam*HI fragment of the *lacZ* gene (14) and the *Hinc*II fragment of the human IFN- β gene (11). The 5-kb fragment corresponds to the MFG-LacZ proviral DNA (A), the 3.5-kb fragment corresponds to the human IFN- β sequence present in the HMB-based vectors (B), and the 20-kb fragment corresponds to the endogenous human IFN- β gene, which serves as an internal control (C). The number of copies per cell was estimated by comparing the intensity of the band to control bands whose intensity corresponds to a known copy number (0.1 or 1 copy). C, untransformed cells; LacZ, MFG-LacZ-infected cells; Δ IFN, HMB-K^bHuIFN β delStyI-transformed cells; IFN, HMB-K^bHuIFN β -transformed cells.

FIG. 4. RT activity in culture media and extracts from cells infected with the defective MFG-LacZ retrovirus. Human T98G (Top), CEM (Middle), and U-937 (Bottom) cells transformed by HMB-K^bHuIFN β delStyI or HMB-K^bHuIFN β were infected with the amphotropic MFG-LacZ retrovirus at a multiplicity of infection close to 1. We have verified that RT activity in culture medium without cells was stable during the experiment (data not shown). RT activity was assayed in triplicate in cell extracts (A) and culture media (B) at various time after infection of untransformed control cells (stippled bars), HMB-K^bHuIFN β delStyI-transformed cells (hatched bars), and HMB-K^bHuIFN β -transformed cells (filled bars). Results are expressed as cpm per 50 μ l of reaction mixture.

transformed cells than in the control cells (Fig. 5), indicating an early block of the infectious cycle.

We therefore examined the possibility that, also with HIV-1, viral entry was decreased. Untransformed and HMB-transformed CEM cells were infected with HIV-1, and RT activity was analyzed in culture medium and intracellularly (Fig. 6). The cellular level of RT activity of human IFN- β -transformed CEM cells remained low after infection, whereas it was detectable 2 hr after infection and reached a maximum 4 hr after infection in the control cells. In the culture medium of the control cells, 6 hr after infection the RT activity had decreased by 15% as compared with the RT activity right after infection at time 0. In contrast, 6 hr after infection, 77% of the original RT activity was still present in the medium of the human IFN- β -transformed CEM cells.

DISCUSSION

Populations of the three different cell types used in our study could be successfully transformed and display SAVE without impeding cell survival and replication. These findings are encouraging for the use of the HMB-K^bHuIFN β amphotropic vector to explore the possibility of developing an anti-HIV-directed somatic-cell gene therapy.

It is our aim to enhance the resistance of lymphocytes and monocytes to HIV infection, and for this reason we have paid particular attention to the antiretroviral effects of low auto-crine human IFN- β production. We found that 6 hr after onset of infection, 40–80% of the infectious particles remained in the culture medium of human IFN- β -transformed

FIG. 5. Detection of HIV proviral DNA in infected CEM cells by Southern blot analysis. Six million CEM cells, untransformed or transformed by HMB-K^bHuIFN β delStyI or by HMB-K^bHuIFN β , were infected with 3×10^5 cpm of the HIV-1_{BRU} stock. One hour later, virus was removed, and cells were washed three times with RPMI 1640 medium containing 0.5 mM EDTA and cultured in fresh culture medium. Genomic DNA was extracted 48 hr later, digested by *Hind*III, and analyzed by Southern hybridization using a probe described by Macé *et al.* (11). The 9-kb fragment corresponds to HIV-1 proviral DNA (A), and the 15-kb fragment corresponds to the endogenous glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene, which serves as an internal control in this experiment (B). C, untransformed CEM cells; HIV, HIV-infected CEM cells; Δ IFN, HMB-K^bHuIFN β delStyI-transformed CEM cells; IFN, HMB-K^bHuIFN β -transformed CEM cells.

cells, whereas only 10% were still present in the culture medium of control cells. That the block prior to reverse transcription was also effective in murine IFN-treated NIH 3T3 mouse cells infected with an ecotropic Moloney-based retrovirus suggests that IFN impairs a general mechanism common to retroviral infection, either at the level of attachment of the virus to its cell-surface receptor or at the level of penetration of the virus core into the cells.

Little is known concerning the molecular mechanism of retroviral entry into host cells. Virus recognition of specific receptors in the host-cell plasma membrane is the first step in infection. The receptor for HIV is the CD4 molecule and the receptor for the ecotropic virus is a cationic-amino-acid

FIG. 6. RT activity in culture medium and extracts from HIV-1-infected CEM cells. Fifty thousand CEM cells, untransformed (stippled bars) or transformed by HMB-K^bHuIFN β delStyI (hatched bars) or by HMB-K^bHuIFN β (filled bars) were infected with 2×10^5 cpm of the HIV-1_{BRU} stock. RT activity is expressed as cpm per 50 μ l of reaction mixture. We studied the stability of the RT activity in culture medium (B) and found a 50% decrease of HIV-1 RT activity 2 hr after infection. Moreover, we found in the extracts (A) of uninfected CEM cells a high level of RT activity at 2 and 6 hr after infection.

transporter (20, 21). The theoretical possibility that down-regulation of viral receptors under the influence of IFN contributes to the lack of infection is unlikely, since the expression of the CD4 HIV receptor was not affected by the low constitutive expression of IFN- β . For numerous viruses, attachment to the receptor is followed by a redistribution or capping of the virus particles on the cell surface. IFN treatment induces an increase in the proportion of cells showing large actin-containing fibers in the form of parallel arrays and an inhibition of the lateral mobility of the cell surface receptors for the lectin concanavalin A, resulting in a significant reduction of capping (22–24). Recently, Delcayre *et al.* (25) have clearly identified inhibition of the capping of the complex formed between Epstein–Barr virus (EBV) and its receptor CD21 as a novel effect of IFN- α in the EBV/B-cell system. Another property of IFN consists in increasing the rigidity of the membrane lipid bilayer (26, 27) through an increase of the cholesterol/phospholipid ratio (28) and an increase of the proportion of saturated relative to unsaturated fatty acid chains (29). The important relationship between the lipid composition of the cell membrane and infection with some viruses has been stressed by Aloia *et al.* (30), who observed a lower cholesterol/phospholipid ratio in plasma membrane isolated from HIV-infected cells as compared with uninfected cells and a higher cholesterol/phospholipid ratio in the HIV membrane than in the host-cell plasma membrane, leading to the concept of cholesterol domains through which HIV evaginates. In this respect, it may be relevant that IFNs can block fusion of the HIV envelope protein and the cellular membrane (31). It has been well established in the murine system that, at the end of the retroviral cycle, budding and maturation are inhibited in IFN-treated cells, suggesting that this could be the consequence of an IFN-induced change in plasma membrane fluidity (2, 32). Quite possibly, a similar mechanism could prevent virus from getting inside the cell, since both processes require fusion of the viral envelope and the plasma membrane.

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