Evidence for a newly discovered cellular anti-HIV-1 phenotype

JAMES H.M. SIMON[1], NATHAN C. GADDIS[3], RON A.M. FOUCHIER[4] & MICHAEL H. MALIM[1,2,4]

Departments of Microbiology[1] and Medicine[2], Cell and Molecular Biology Graduate Group[3], Howard Hughes Medical Institute[4], University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6148, USA
Correspondence should be addressed to M.H.M.; email: malim@hhmi.upenn.edu

Animal cells have developed many ways to suppress viral replication, and viruses have evolved diverse strategies to resist these. Here we provide evidence that the virion infectivity factor protein of human immunodeficiency virus type 1 (HIV-1) functions to counteract a newly discovered activity in human cells that otherwise inhibits virus replication. This anti-viral phenotype is shown by human T cells, the principal *in vivo* targets for HIV-1, and, based on our present understanding of virion infectivity factor action, is presumed to act by interfering with a late step(s) in the virus life cycle. These observations indicate that the inhibition of virion infectivity factor function *in vivo* may prevent HIV-1 replication by 'unmasking' an innate anti-viral phenotype.

Several mechanisms of non-immune, cell-mediated resistance to retroviral infection have been described. These include *Fv-1* resistance of murine cells to infection by murine leukemia virus[1], and the recently described polymorphism in the CCR-5 chemokine receptor (*CCR5*-Δ32) that confers resistance to infection by certain human immunodeficiency virus type-1 (HIV-1) strains[2,3]. In addition, polymorphisms in the genes encoding the CCR2 chemokine receptor or the stromal-derived factor (SDF-1) chemokine correlate with delayed progression to AIDS in individuals infected with HIV-1[4,5]. Here we have identified what seems to be a previously unrecognized form of cellular resistance to viral infection, by examining the interplay between human cells and the HIV-1 virion infectivity factor (Vif) regulatory/accessory protein. This activity results in the inhibited replication of HIV-1 and other retroviruses, such as simian immunodeficiency virus (SIV) and murine leukemia virus[6], but is overcome by expression of the HIV-1 Vif protein.

Vif has an essential role during the lentiviral life cycle, and its expression is required for robust infection of cultured primary cells, cell lines or animals[6–15]. For HIV-1, Vif is also dispensable for replication in many diverse cell lines[7,9,10,12,16–19]. Accordingly, cells have been divided into groups based on their ability to support the replication of *vif*-deficient (Δ*vif*) HIV-1. Non-permissive cells, such as HUT78, H9 or peripheral blood lymphocytes, are resistant to spreading infection by Δ*vif* viruses, and produce Δ*vif* viruses that are 10- to 100-fold less infectious than wild-type HIV-1[9,17,19–21]. In contrast, permissive cells, such as CEM-SS, C8166, HeLa, COS or 293T, produce Δ*vif* viruses that have infectivities indistinguishable from those of wild-type viruses. Two possible explanations for these phenomena are that permissive cells express an activity (or factor) that can compensate for Vif, or that non-permissive cells have an activity that manifests itself as an inhibition of HIV-1 infectivity in the absence of Vif.

One experimental approach to evaluate such hypotheses is to investigate the infectivity phenotype of HIV-1/Δ*vif* produced from transient heterokaryons formed between permissive and non-permissive cells. If the patterns of gene expression responsible for these parental cell phenotypes are maintained in the heterokaryons, two potential outcomes are possible. If the permissive phenotype is retained, with Vif not being required for the infectivity of viruses produced from cell fusions, perhaps permissive cells express an activity that can substitute for Vif. In contrast, should the heterokaryons display the non-permissive phenotype, with full viral infectivity being dependent on the expression of Vif, perhaps non-permissive cells express an activity that leads to the loss of HIV-1 infectivity but is subject to Vif-mediated suppression.

To identify appropriate permissive and non-permissive parent cell lines for these studies, we tested the infectivities of wild-type HIV-1 and HIV-1/Δ*vif* produced from HUT78, CEM-SS or 293T cells. Viruses produced from these cells were normalized according to reverse transcriptase (RT) activity and used to infect the C8166/HIV-CAT indicator cell line (Fig. 1). As demonstrated previously, CEM-SS and 293T cells were fully permissive, in that both produced wild-type and Δ*vif* viruses that were similarly infectious. In contrast, HUT78 cells were non-permissive, as the Δ*vif* virus produced by these cells was about 15-fold less infectious than its wild-type counterpart.

To produce virions from transient heterokaryons formed by the fusion of non-permissive and permissive cells, we devised a *trans*-complementation strategy in which, before fusion, one of the parental cell lines expressed *env*-deficient cores (HIV-1/Δ*env*), whereas the other expressed the HIV-1 Env glycoprotein but no viral cores (Fig. 2). This strategy ensured that infectious virions (those that had both cores and Env) were produced exclusively by heterokaryons and not by either one of the parental cell lines. In the Vif-expressing heterokaryons, Vif was supplied by either the core-expressing T cells or the Env-expressing 293T cells.

Core-producing CEM-SS (permissive) or HUT78 (non-permissive) cells were prepared by initial infection with pseudotyped Vif-expressing or Δ*vif* versions of HIV-1/Δ*env*, whereas their 293T (permissive) fusion partners were prepared by co-transfection with an HIV-1 Env expression vector and either a Vif expression vector or its Δ*vif* derivative. Heterokaryon formation was then initiated by mixing cultures of core-producing T cells and Env-expressing 293T cells, and was mediated by the interaction of CD4/CXCR4 on the surface of the T cells with Env on the surface of 293T cells. This approach proved to be very efficient at inducing T cell–293T cell fusion, in that most of the T cells had visibly fused within 24 hours of cell mixing (data not shown). As a control for confirming that fusion efficiencies were consistent within a data set, the 293T cultures were additionally transfected with a plasmid carrying an HIV-1 long ter-

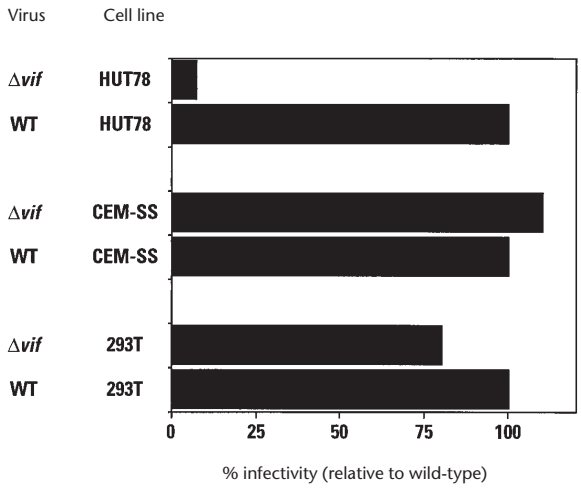

Fig. 1 Vif is required for HIV-1 infectivity in HUT78 cells, but not in CEM-SS or 293T cells. HUT78, CEM-SS and 293T cells were infected with VSV-G pseudotyped stocks of HIV-1 or HIV-1/Δ*vif*. Progeny viruses were collected at 30 h and used to infect C8166/HIV-CAT indicator cells. At 24 h, levels of CAT in the cell lysates were determined and used as a measure of virus infectivity. The background CAT activity of mock-infected cells was subtracted from all values, and the relative infectivity of each Δ*vif* virus is shown relative to a value of 100% for the corresponding wild-type (WT) viruses.

minal repeat (LTR)/CAT cassette; the formation of heterokaryons therefore resulted in a substantial induction of LTR-driven CAT activity by Tat that was derived from the core-producing T cells. CAT expression was dependent on virus expression in the T cells and Env in the 293T cells, but was equivalent irrespective of the expression or source of Vif (data not shown).

Progeny viruses from the culture supernatants were collected and normalized for RT activity 24 hours after the initiation of heterokaryon formation, and their infectivities were determined using C8166/HIV-CAT cells (Fig. 3). As expected, the viral particles that were being produced by T cells in cell mixtures that lacked Env were non-infectious; this confirmed that the pseudotyped viruses that had initially been used to prepare the core-producing T cells had been removed from the cultures. As might have been expected, when both cell components of the heterokaryons were permissive (CEM-SS and 293T), the infectivities of Env-containing virions were always high and were not affected by the presence of Vif. However, when non-permissive HUT78 cells were fused to 293T cells, the virions that were produced in the absence of Vif were about 10-fold less infectious than those produced in its presence; this happened whether Vif was derived from the core-producing HUT78 cells or the Env-expressing 293T cells.

Although these data are consistent with the idea that non-permissive cells have an activity whose presence somehow prevents the production of infectious virus in the absence of Vif, it was also possible that a compensating activity in 293T cells could have been extinguished after cell fusion. However, the extinction of gene expression in transient heterokaryons is often relatively minor until 24 hours after fusion, and can be sensitive to modest changes in parent cell ratios[22,23].

We did two experiments to address the possibility of extinction. We collected viruses from heterokaryons formed between Env-expressing 293T cells and Vif-expressing or Δ*vif* core-producing HUT78 cells at various times after cell mixing and examined them for infectivity (Fig. 4*a*). If extinction of a compensating activity had been occurring, the infectivity of Δ*vif* virions relative to wild-type virions would be expected to be higher at early time points and then to decline. This trend was not observed at the 12- and 24-hour timepoints; thus, a compensatory activity probably was not being lost after fusion (the time-dependent increases in the infectivity of viruses was presumably due to increasing proportions of virions being derived from Env-expressing heterokaryons). We also determined the infectivities of viruses produced from heterokaryons in which the ratio of HUT78 cells to 293T cells was varied from 2:1 to 1:4 (Fig. 4*b*). Here, the Vif-mediated increases in infectivity were similar regardless of cell ratio; had extinction of a compensating factor been occurring, the Δ*vif* viruses from cell mixtures with greater numbers of permissive 293T cells might have been predicted to have infectivities substantially closer to those of their Vif-expressing counterparts. These results support the hypothesis that the non-permissive phenotype of HUT78 T cells is dominant over the permissive phenotype of 293T cells.

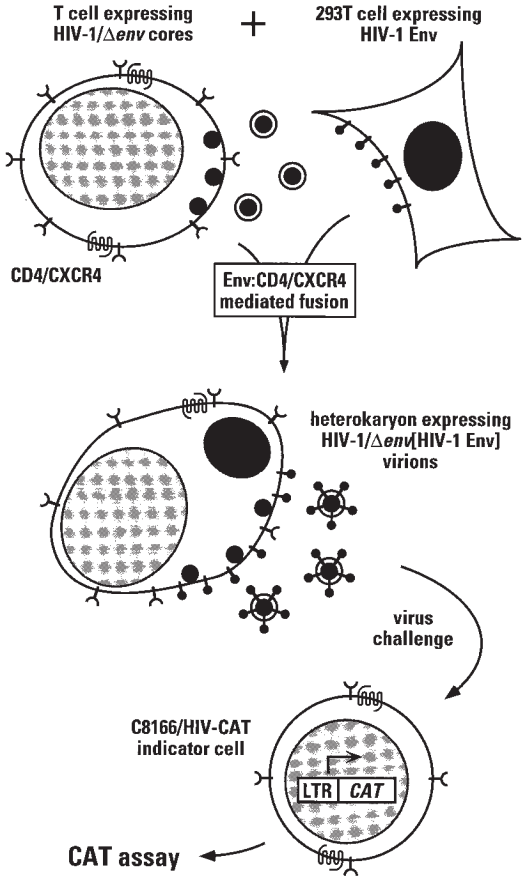

Fig. 2 Strategy for the production of HIV-1 from transient T cell–293T heterokaryons. T cells (HUT78 or CEM-SS) were initially infected with HIV-1/Δ*env* or HIV-1/Δ*vif*Δ*env* pseudotyped with VSV-G. These core-producing T cells were mixed with Env-expressing 293T cells such that CD4/CXCR4 would interact with Env and facilitate efficient cell–cell fusion. Because the heterokaryons contained both HIV-1/Δ*env* and HIV-1 Env, new progeny virions (HIV-1/Δ*env*[HIV-1 Env]) were rendered capable of fusing to target cells, such as C8166/HIV-CAT indicator cells, and of initiating productive infections.

Discussion

Our findings are most consistent with the idea that non-permissive human T cells have an activity whose expression presence results in

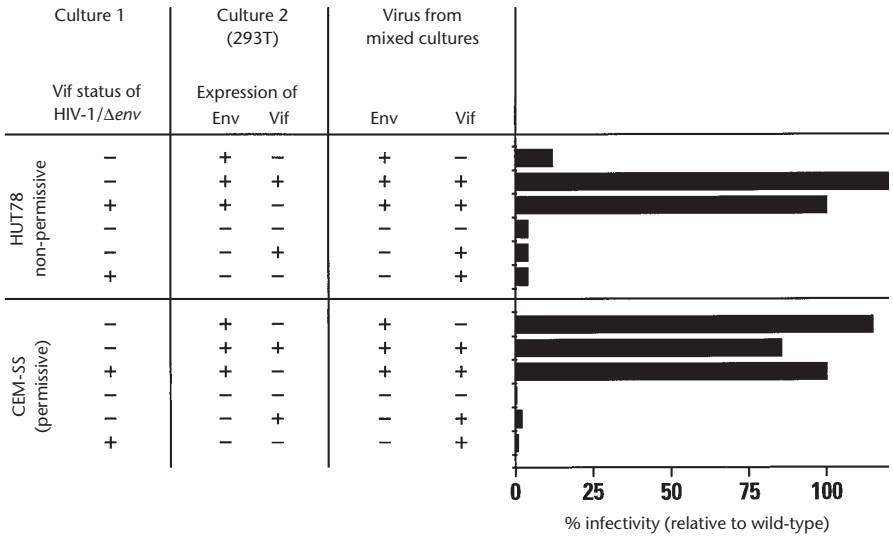

Fig. 3 Infectivities of HIV-1/Δ*vif* and HIV-1 produced from heterokaryons. HUT78 or CEM-SS cells (Culture 1) expressing HIV-1/Δ*env* or HIV-1/Δ*vif*Δ*env* cores were mixed with an equal number of 293T cells (Culture 2) that had been transfected with an Env expression vector and a Vif expression vector, or corresponding negative control vectors; the presence (+) or absence (–) of Env and Vif in each culture before and after mixing is indicated. Viruses collected at 24 h were used to challenge C8166/HIV-CAT cells, and the levels of infectivity were determined by CAT assay. The CAT activity of mock-infected cells was subtracted from all values, and the infectivity of each virus is shown relative to the 100% infectivity of the Env-plus viruses obtained using Vif-expressing T cells as core-producers. The data represent the mean of four independent experiments (HUT78/+Env samples), three experiments (HUT78/–Env and CEM-SS/+Env samples) or two experiments (CEM-SS/–Env samples). The relative infectivities with standard deviations for the HUT78/+Env viruses are: no Vif, 12 ± 3.3; Vif from 293T cells, 123 ± 18; Vif from HUT78 cells, 100.

the inhibition of HIV-1 infectivity, and that the function of Vif is to counteract this. Vif increased virus infectivity by 10- to 20-fold in experimental conditions in which viruses were produced either by unfused non-permissive cells (Fig. 1) or by non-permissive cells fused to permissive cells (Figs. 3 and 4). This activity is not restricted to HIV-1, as the infectivities of other primate lentiviruses (HIV type-2 and various SIVs) as well as murine leukemia virus, an oncoretrovirus, can be modulated by expression of HIV-1 Vif in human T cells[6,12,13]. At present, it is unknown whether this putative inhibitor function is constitutively present in non-permissive cells or is expressed in response to viral infection. We speculate that this activity presumably disrupts (either directly or indirectly) one of the stages of the life cycle that are thought to be modulated by Vif: assembly, budding and/or maturation[9,10,19,24,25].

There are many examples of anti-viral host defense activities being modified by specific viral gene products. Many of these relate to immune and inflammatory responses and frequently involve apoptosis[26–28]. For example, activation of signaling cascades by engagement of members of the tumor necrosis factor receptor superfamily is integral to many anti-viral responses. Many viruses are able to interfere with signaling through these pathways by a variety of mechanisms involving secreted as well as intracellular proteins; for example, poxviruses encode secreted receptor-like proteins that impede receptor–ligand interactions[29], as well as non-secreted proteins that antagonize protein–protein interactions essential to signal transduction[30–32]. The anti-viral activities of interferon-α are also well documented, and involve the induction of both double-stranded RNA-dependent protein kinase (PKR) and 2′5′-oligoadenylate (2′5′A) synthetase (ref 33). To counteract the translational inhibitory effects of PKR, adenovirus encodes a structured RNA, VAI RNA, which binds to PKR and prevents its activation[34], whereas poxviruses express proteins that serve as decoy substrates for PKR or

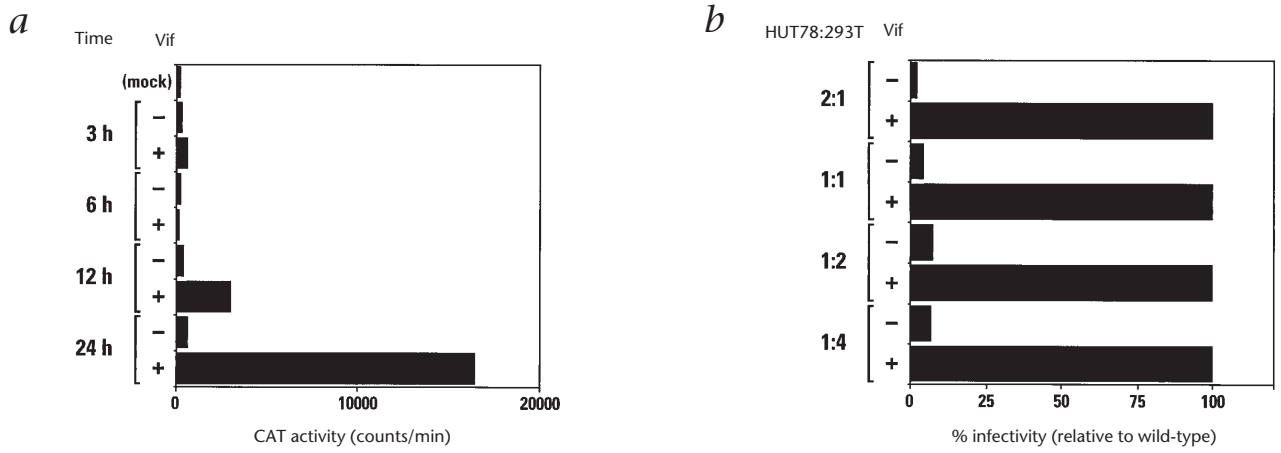

Fig. 4 Effects of time and cell ratio on Vif function in heterokaryons. ***a***, Heterokaryons were formed between equal numbers of Env-expressing 293T cells and HIV-1/Δ*env* or HIV-1/Δ*vif*Δ*env* core-producing HUT78 cells. Viruses were collected at various times thereafter and the levels of infectivity were determined; because many of the CAT values were close to that of the mock-infection control, the actual levels of CAT activity (in counts per min) are shown rather than relative infectivities. ***b***, Viruses were collected 24 h after wild-type or Δ*vif* core-producing HUT78 cells were mixed at the indicated ratios (far left column) with 2×10^6 Env-expressing 293T cells. The background CAT activity of mock-infected cells was subtracted from all values, and the relative infectivity of each Δ*vif* virus is shown relative to a value of 100% for the corresponding wild-type (WT) viruses. Mean values from two experiments are shown.

bind to dsRNA directly[29]. A very different, and apparently unique, form of cellular resistance has been described for the murine *Fv-1* locus and murine leukemia virus infection[1]. Unlike the Vif effect, *Fv-1* restriction occurs at an early post-entry step and is mediated by a host gene that is distantly related to the *gag* region of a human endogenous provirus-like element[35]. Infectivity can be restored by mutations in the capsid portion of the viral *gag* gene rather than by a specialized viral gene product[36]. These examples all seem to be very different from the Vif requirement that is imposed by non-permissive cells. Here, viral infectivity is qualitatively different during virus production, rather than viral output being quantitatively reduced or infection being restricted after entry into target cells.

Our results not only demonstrate the existence of a potentially newly discovered mode of host cell resistance to viral replication, but also indicate that Vif may represent a useful future target for therapeutic intervention in HIV-1 infections and AIDS. Specifically, as human T cells naturally have an activity that renders HIV-1 virions non-infectious in the absence of Vif, blockade of Vif function should allow this putative anti-viral activity to become operative.

Methods

Viruses and virus-producing cells. Cultures expressing HIV-1 (wild-type virus), HIV-1/Δ*vif*, HIV-1/Δ*env* or HIV-1/Δ*vif*Δ*env* were prepared by single-cycle, high-titer challenge using stocks derived by co-transfection of 293T monolayers with the pIIIB, pIIIB/Δ*vif*, pIIIB/Δ*env* or pIIIB/Δ*vif*Δ*env* proviruses[12], respectively, and an expression vector for the G glycoprotein of vesicular stomatitis virus, pHIT/G (ref. 6). The *env*-deficient proviruses were constructed by deletion of the *env* sequences between nucleotides 7,039 and 7,619 from pIIIB or pIIIB/Δ*vif*. Cells were incubated with virus for 4 h, washed extensively with phosphate-buffered saline to eliminate input virus, and incubated in fresh medium for 24–36 h. Virus-containing culture supernatants were clarified by centrifugation at 500*g* for 5 min, passed through filters with 0.45 μm pores, and quantitated using a reverse transcriptase (RT) assay[6].

Virus infectivity assays. Virus preparations from a given experiment were normalized for RT activity and were used to infect 0.5×10^6 C8166/HIV-CAT cells, and the expression of chloramphenicol acetyl transferase (CAT) was determined after 24–48 hours[19]. Because these indicator cells have the CAT gene under the transcriptional control of the HIV-1 long terminal repeat (LTR), CAT expression is induced by Tat after the expression of newly formed proviruses, and therefore serves as a quantitative measure of virus infectivity.

Formation of heterokaryons. Unless stated otherwise, transient heterokaryons were formed by adding about 2×10^6 HIV-1/Δenv (Vif expressing or Δvif)-producing T cells to about 2×10^6 adherent 293T cells that had been transfected 36 h previously with 1 μg of the HIV-1 Env expression vector pSRHS (ref. 37)(or a negative control vector), 3 μg of the Vif expression vector pLN-M/hVif (ref. 12)(or its Δvif counterpart), and 0.3 μg of the HIV/CAT vector GV3LCATs (ref. 38). Cell mixtures were maintained in 4 ml medium for 24 h (by which time about 80 % of the T cells had visibly fused), virus stocks were collected, and RT and infectivity assays were done. Whole-cell lysates were also prepared at the time of collection, and the levels of CAT expression determined to verify consistent cell–cell fusion efficiency.

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