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The CC-Chemokine RANTES Increases the Attachment of Human Immunodeficiency Virus Type 1 to Target Cells via Glycosaminoglycans and Also Activates a Signal Transduction Pathway That Enhances Viral Infectivity

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We have studied the mechanisms by which the CC-chemokine RANTES can enhance the infectivities of human immunodeficiency virus type 1 (HIV-1) and other enveloped viruses, when present at concentrations in excess of 500 ng/ml in vitro. Understanding the underlying mechanisms might throw light on fundamental processes of viral infection, in particular for HIV-1. Our principal findings are twofold: firstly, that oligomers of RANTES can cross-link enveloped viruses, including HIV-1, to cells via glycosaminoglycans (GAGs) present on the membranes of both virions and cells; secondly, that oligomers of RANTES interact with cell-surface GAGs to transduce a herbinycin A-sensitive signal which, over a period of several hours, renders the cells more permissive to infection by several viruses, including HIV-1. The enhancement mechanisms require that RANTES oligomerize either in solution or following binding to GAGs, since no viral infectivity enhancement is observed with a mutant form of the RANTES molecule that contains a single-amino-acid change (glutamic acid to serine at position 66) which abrogates oligomerization.

Infection of target cells by human immunodeficiency virus type 1 (HIV-1) is mediated by interactions of the viral envelope glycoproteins with CD4 and a coreceptor (32). Among the latter, the CC-chemokine receptor CCR5 and the CXC-chemokine receptor CXCR4 are the most physiologically important (8, 27, 56). The cognate chemokines can influence HIV-1 infection in several ways in vitro, the most commonly observed being inhibition of virus entry because of competition between the virus and the chemokine for binding sites on the same receptor (5, 17, 22, 29, 64, 66, 80, 84, 85, 94). Receptor down-regulation in response to chemokine binding and signaling can also interfere with virus entry by reducing the density of available coreceptors on the cell surface (2, 4, 51). However, CC-chemokines have also been reported to enhance HIV-1 infection of various cells in vitro (26, 36, 45, 58, 78).

Previously, we showed that the CC-chemokine RANTES could enhance HIV-1 infection of target cells in a manner that was independent of CD4 and any known coreceptor and even independent of the route of virus entry (36). Other CC-chemokines, such as macrophage-inhibitory protein (MIP)-1α and MIP-1β, did not have this effect (36). The extent of infectivity enhancement caused by RANTES was significant: in excess of 100-fold under some conditions. Two components of the enhancement mechanism were noted: one was apparent when the target cells were preincubated for several hours with RANTES prior to the addition of virus, and the other was evident when RANTES was added simultaneously with the virus (36). Here, we further analyze how RANTES can increase viral infectivity. We conclude that a major mechanism of infectivity enhancement is caused by the cross-linking of virions to the cell surface by oligomers of RANTES. These oligomers form after binding to glycosaminoglycans (GAGs), such as heparan sulfate, on both the virion and cell membranes. Of note is the fact that RANTES variants that do not oligomerize do not enhance viral infectivity (24). A second mechanism of viral infectivity enhancement arises from the prolonged interaction of RANTES with cell surface GAGs, which activates a herbinycin A-sensitive, tyrosine-kinase-dependent signal transduction pathway.

MATERIALS AND METHODS

Cells. HeLa-CD4 cells were provided by David Kabat (47). They were maintained in Dulbecco’s minimal essential medium containing 10% fetal calf serum (FCS); glutamine, and antibiotics and split twice a week. Chinese hamster ovary (CHO)-K1 cells, heparan sulfate-mutant CHO cells (psgA-745 cells), and chondroitin sulfate-mutant CHO cells (psgA-745 cells) were all obtained from the American Type Culture Collection (Rockville, Md.) (30, 31, 49). These lines were maintained in F12K nutrient mixture (Kightl’s modification) supplemented with 10% FCS.

Chemokines. Human MIP-1α was purchased from R&D Systems Inc. (Minneapolis, Minn.). Recombinant human RANTES was produced in the bacterial host Escherichia coli as previously described (75). AOP-RANTES was derived from RANTES, as reported elsewhere (80). Rat RANTES was purchased from Peprotech Inc., Rockville, N.J. RANTES(3-68) was made by total peptide synthesis and provided by RMP DICTAGENE, Epalinges, Switzerland. The RANTES(3-68) molecule has the wild-type RANTES sequence but lacks the two N-terminal amino acids, serine and proline (65, 74). Its N-terminal sequence is therefore YSSDTPP. . . . The mutated, nonaggregating RANTES molecule, BB-10520 RANTES, was made at British Biotechnology Ltd. (Oxford, United Kingdom) (24). It has the wild-type RANTES sequence except for a single-amino-acid change: glutamic acid to serine at residue 66 (E66→S). The RANTES E66→S gene was expressed and secreted from the yeast Pichia pastorias at high yield. The purified protein, designated BB-10520 RANTES, had undergone truncation of the two N-terminal amino acids so that its N-terminal sequence was YSSDTPP. . . . (24). This molecule and RANTES(3-68) are therefore identical except for the E66→S substitution.

Viruses. Env-pseudotyped, luciferase-expressing reporter viruses were produced by the calcium phosphate technique (15, 23, 29). The 293 T cells were cotransfected with the envelope-deficient HIV-1 NL4-3 construct, pNL-Luc, and with a pSV vector expressing viral envelope glycoproteins (15, 23, 29). The
pNL-Luc virus carries the luciferase reporter gene; the pSV vectors express envelope glycoproteins derived from HIV-1, amphotropic murine leukemia virus (MuLV), or vesicular stomatitis virus (VSV). The Env-pseudotyped viruses are designated as HIV-1-R5, HIV-1-MuLV, etc., with the subpect ase representing the pseudotyped enve gene.

**Viral infection assay with luciferase readout.** The extent of HIV-1 entry was determined by using a single-cycle infection assay (15, 23, 29). One day before counter, and the data were plotted as described elsewhere (37).

For experiments in which RANTES was added to target cells simultaneously with the virus (Fig. 1a) or simultaneously with the virus (Fig. 1b) (36). Significant, but somewhat reduced, infectivity enhancement was also observed with RANTES(3-68), a variant of RANTES that is N-terminally truncated by two residues (Fig. 1). In contrast, BB-10520 RANTES, which differs from the conventional RANTES molecule at only a single position (E66>S) and by a two-residue truncation at the N terminus (24), caused a modest decrease in HIV-1_MuLV infectivity when added to the cells 24 h prior to the virus (Fig. 1a) or simultaneously with the virus (Fig. 1b) (36).

Clearly, whether viral infectivity enhancement occurs is a function of the RANTES sequence, which presumably affects an important structural feature of this molecule. Of note is the fact that AOP-RANTES and rat RANTES both behave like the wild-type human RANTES molecule in that they also enhance viral infectivity (references 24 and 36 and data not shown). Each of these molecules, and also RANTES(3-68), is identical to wild-type human RANTES at residue 66, but each differs from BB-10520 RANTES at this position. The two-residue N-terminal truncation of BB-10520 RANTES is not responsible for its inability to enhance viral infectivity, since RANTES(3-68) has the same truncation yet still causes infectivity enhancement (Fig. 1).

**RESULTS**

Enhancement of viral infectivity by RANTES is dependent on the RANTES sequence. We have demonstrated previously that RANTES, but not other CC-chemokines such as MIP-1α and MIP-1β, is able to enhance viral infectivity independently of the envelope glycoproteins and the route used by the test viruses to enter target cells (36). To gain further insight into the underlying mechanisms, we tested several different sequence variants of the RANTES molecule. The origins and properties of these RANTES variants are described in Materials and Methods. To eliminate any direct influences of RANTES on the receptor used for virus entry, we used as a test virus MuLV envelope glycoprotein pseudotypes of HIV-1 (HIV-1_MuLV); the entry of HIV-1_MuLV occurs via a plasma membrane phosphate transporter which is not known to be a RANTES receptor (53). As target cells, we used the HeLa-CD4 cell line, since RANTES enhances HIV-1_MuLV infection of HeLa cells just as it does HIV-1 infection via CD4 and the CC or CXCR4 coreceptors (36).

Based on our previous observations, RANTES enhancement of HIV-1_MuLV infectivity was studied in two ways (36). The target cells were pretreated with CC-chemokines for 24 h and then washed before the addition of virus for 2 h in the absence of CC-chemokine (Fig. 1a); alternatively, CC-chemokines were added to the cells for a 2-h period, simultaneously with the viral inoculum, and then washed away (Fig. 1b).

As was found previously, RANTES but not MIP-1α substantially enhanced HIV-1_MuLV infectivity in a dose-dependent manner, whether it was added to the target cells 24 h prior to the virus (Fig. 1a) or simultaneously with the virus (Fig. 1b) (36). Significant, but somewhat reduced, infectivity enhancement was also observed with RANTES(3-68), a variant of RANTES that is N-terminally truncated by two residues (Fig. 1). In contrast, BB-10520 RANTES, which differs from the conventional RANTES molecule at only a single position (E66>S) and by a two-residue truncation at the N terminus (24), caused a modest decrease in HIV-1_MuLV infectivity when added to the cells 24 h prior to the virus (Fig. 1a). Furthermore, BB-10520 RANTES stimulated only a very slight increase in infectivity when added at the highest concentration tested (10 μg/ml) simultaneously with HIV-1_MuLV (Fig. 1b).
increase in the infectivity of cell-free virus when it is present during the virus-cell adsorption phase of the viral life cycle. One explanation of this would be that RANTES promotes virion binding to the cells. To test this directly, we measured the attachment of HIV-1IIIB to target cells (Fig. 2), using an assay developed by Mandor, Ugolini, and colleagues (54, 88). Virions grown in cells expressing HLA-DR (e.g., H9 cells) incorporate this protein into their membranes on budding from the cell membrane. The virions are then added to cells that do not express HLA-DR (e.g., A2.01 or A3.01 cells), and bound HLA-DR (i.e., virion membrane derived) is detected by FACS, using an anti-HLA-DR antibody. Cells that grow in suspension were used for this assay, to avoid damage to the membrane composition of adherent cells when they are detached for FACS analysis. By using CEM.NKR cells that are closely related to A3.01 cells, we have confirmed that RANTES causes viral infectivity enhancement in T cells which grow in suspension (data not shown).

Because cells produce small vesicles derived from cell membranes that are of a size similar to that of virions, it is necessary to control for the binding of these vesicles to target cells (9, 35). Consequently, we used sucrose gradient-purified preparations from both HIV-1IIIB-infected and uninfected H9 cells, designated IIIIB/H9 vesicles and H9 vesicles, respectively. The former contain virions and microvesicles; the latter contain only microvesicles.

The binding of virions and microvesicles to CD4-positive A3.01 cells was measured in the presence and absence of RANTES and BB-10520 RANTES (Fig. 2). In one set of experiments, we mimicked what happens when RANTES and virions are added simultaneously to target cells in infectivity assays (Fig. 1b and 2a); in a second set, we treated the cells for 24 h with the RANTES molecules before adding virions in the absence of chemokine, again mimicking what happens in some infectivity assays (Fig. 1a and 2b).

We could detect the binding to A3.01 cells of HIV-1IIIB virions (IIIIB/H9 vesicles) and, to a lesser extent, of the control microvesicles (H9 vesicles) (Fig. 2). The simultaneous addition of RANTES (5 µg/ml) caused a substantial increase in the number of A3.01 cells to which virions or microvesicles were attached. The effect of RANTES was greater with the virion-containing preparation than with the microvesicles, especially at a RANTES concentration of 1 µg/ml (Fig. 2a). In contrast, BB-10520 RANTES only slightly increased the number of cells that had virions attached and only at the highest concentration tested (5 µg/ml). BB-10520 RANTES had no effect on microvesicle attachment (Fig. 2a). Furthermore, when virion attachment was quantified by measuring the median fluorescence intensity levels, as opposed to the percentage of fluorescence-positive cells, BB-10520 RANTES caused no increase whereas the same concentration of RANTES raised the median fluorescence intensity by 20-fold (data not shown).

Analogous experiments were performed with CD4-negative A2.01 cells. In the absence of RANTES, no binding of either the IIIIB/H9 virions or the control H9 vesicles was detectable (data not shown). This is consistent with previous observations that CD4 is required on the target cells for specific, stable virus attachment in this assay (54, 88). However, RANTES, either added simultaneously with the virions or used to pretreat the cells, caused a large increase in the binding of both virions and microvesicles to the A2.01 cells (data not shown). These effects of RANTES were not mimicked by BB-10520 RANTES (data not shown). Thus, RANTES can promote the attachment of virions and cell membrane-derived vesicles to target cells in a CD4-independent manner.

RANTES binds to virions. One mechanism by which RANTES could promote virus-cell attachment would be for it to bind simultaneously to both virions and cells, cross-linking the former to the latter. It is well established that RANTES binds to cell surfaces via multiple receptors, including both classical chemokine receptors (73, 92) and GAGs (10, 37, 66, 89). To test whether RANTES can also bind to virions, we used two assays. In the first, we attached RANTES to magnetic beads via indirectly adsorbed anti-RANTES antibodies and then added HIV-1IIIB and determined the extent of virion binding by measuring how much HIV-1 p24 antigen was associated with the beads. There was significantly greater binding of virions to RANTES-coated beads than to control beads lacking RANTES (Fig. 3a). Virion attachment to the RANTES-coated beads was inhibited by the soluble GAG chondroitin sulfate,
suggesting that the process was GAG mediated (see Fig. 6). The BB-10520 RANTES molecule did not promote significant virion attachment to beads (Fig. 3a), despite being able to recognize the anti-RANTES monoclonal antibody coating the beads (data not shown). In a similar assay, RANTES and MIP-1β were immobilized on streptavidin-coated beads via biotinylated polyclonal antibodies. The amount of virus captured on the beads was 20-fold greater in the presence of RANTES than when MIP-1β was used (Fig. 3b), which is consistent with the ability of RANTES, but neither MIP-1α nor MIP-1β, to enhance viral infectivity (36) (Fig. 1).

**RANTES forms oligomers induced by binding to GAGs.** The results discussed above demonstrate that RANTES molecules which enhance viral infectivity promote virion attachment to cells and also bind to virions. In contrast, a RANTES variant (BB-10520 RANTES) that does not enhance virus-cell attachment also does not cause virus-cell binding and is not virion reactive. This correlation suggests that RANTES cross-links viruses to cells by binding simultaneously to receptors present on both the virion and cell membranes. But does a single RANTES molecule cause cross-linking or must RANTES oligomerize? To test this, we measured the extent to which RANTES and its variants can oligomerize, using an assay in which the RANTES molecules that bind to immobilized heparin, a typical GAG, are quantitated (Fig. 4).

![FIG. 3. RANTES binds to virions. (a) RANTES was captured on magnetic beads via an anti-murine immunoglobulin G antibody and a murine anti-RANTES antibody (solid bars). HIV-1_MuLV virions were then reacted with the beads for 2 h in the presence or absence of the indicated concentrations of soluble chondroitin sulfate (CS). The use of BB-10520 RANTES is indicated by the shaded bar, and the background binding in the absence of RANTES is represented by an open bar. The extent of virion capture was measured by p24 antigen determination and is expressed as the percentage of that achieved in the presence of RANTES but absence of CS (6.5 ng/sample, defined as 100%). The data shown are from one of two to three independent experiments. (b) Biotin-labeled antibodies to CC-chemokines were immobilized on streptavidin-coated magnetic beads and incubated with the appropriate CC-chemokines (solid bars, RANTES; shaded bars, MIP-1β) before the addition of HIV-1_MuLV virions for 2 h. The extent of virion capture was measured by p24 antigen determination. The data shown are from one of three independent experiments.](http://jvi.asm.org/content/jvi/73/10/6373/F3)

FIG. 2. RANTES promotes virion adsorption to target cells. (a) A3.01 cells were incubated for 2 h on ice with preparations containing HIV-1 IIIB virions (IIIB/H9 vesicles) or control microvesicles (H9 vesicles) in the presence of the indicated concentrations of RANTES (hatched bars) or BB-10520 RANTES (shaded bars) or with no CC-chemokine (solid bars). (b) A3.01 cells were treated for 24 h at 37°C with RANTES, BB-10520 RANTES, or no CC-chemokine, as described above. IIIB/H9 vesicles or control H9 vesicles were subsequently added for 2 h on ice in the absence of CC-chemokine. In both panels, cell-bound particles were detected by FACS after HLA-DR monoclonal antibody staining. The values shown are the percentages of positive cells that were gated.

![FIG. 4.](http://jvi.asm.org/content/jvi/73/10/6373/F4)
Qualitatively, there is a correlation between the abilities of RANTES-based molecules to oligomerize and to enhance viral infectivity (Fig. 1 and 4). Quantitatively, the concentration range at which RANTES binding to immobilized heparin occurs should not be precisely compared with what happens when RANTES binds to the cell surface, because the efficiency of the latter varies with the cell surface GAG composition and concentration. Overall, the results shown in Fig. 4 are consistent with the hypothesis that RANTES oligomers attach simultaneously to both virions and cells, cross-linking one to the other and promoting viral infectivity by increasing the amount of cell-bound virus.

GAGs are involved in the attachment of virions to cells via RANTES. To what receptor(s) on virions and cells do oligomers of RANTES bind? We reported previously that we could not identify a seven-transmembrane-spanning receptor common to all the human and nonhuman cell lines in which RANTES enhanced viral infectivity (36). These negative findings, together with knowledge of the RANTES concentration range over which enhancement occurred, focused our attention on cell surface GAGs. These molecules, typified by heparan sulfate and chondroitin sulfate, are known to be low-affinity cell surface RANTES receptors (10, 37, 66). Indeed, RANTES is secreted from CD8\(^+\) cells as GAG complexes (89).

To address the involvement of GAGs, we first used two cell lines defective in GAG synthesis, derived by treating wild-type CHO-K1 cells with a chemical mutagen (31, 49). These CD4-negative lines have been used to demonstrate that GAGs are required for adhesion of the malarial circumsporozoite protein to target cells (34). The pgsA-745 line contains a mutation resulting in a defect in xylosyltransferase, an enzyme that attaches xylose to a serine residue of the core protein in the first sugar transfer reaction of GAG synthesis (31). This cell line does not, therefore, produce any GAGs. The pgsD-677 line expresses altered forms of N-acetylglucosaminyltransferase and glucuronosyltransferase, enzymes required for heparan sulfate polymerization. These cells specifically lack heparan sulfate and accumulate three- to fourfold more chondroitin sulfate than wild-type cells (49).

Because HIV-1 and HIV-1\(_{MuLV}\) pseudotypes do not efficiently infect CHO-K1 cells (41) (or mutants thereof), we used HIV-1 pseudotyped with the VSV envelope glycoproteins (HIV-1\(_{VSV}\)): RANTES enhances the infectivity of this virus just as it does HIV-1 and HIV-1\(_{MuLV}\) (36). The enhancement of HIV-1\(_{VSV}\) infectivity by RANTES was significantly reduced in simultaneous-addition experiments with both CHO-K1 cell mutants, especially with the pgsA-745 GAG-deficient cell line (Fig. 5b). Similar, but more pronounced, reductions in the extent of infectivity enhancement were observed when both mutant CHO-K1 cell lines were pretreated with RANTES for 24 h before the addition of HIV-1\(_{VSV}\) in the absence of RANTES compared to what was observed with the wild-type CHO-K1 cells. Indeed, there was no significant enhancement of infectivity with the pgsA-745, GAG-deficient cells under these conditions (Fig. 5a).

These results implicate cell surface GAGs as mediators of RANTES-induced viral infectivity enhancement. The most likely explanation of the effect is that oligomers of RANTES bind to GAGs on both the virus and cell membranes, cross-linking the two. We investigated whether this was, in fact, the case by adding soluble GAGs as competitors for RANTES binding to virion- or cell-associated GAGs. When HeLa-CD4 cells were treated with RANTES (5 \(\mu\)g/ml) in the presence or absence of soluble GAGs for 24 h prior to the addition of HIV-1\(_{MuLV}\), both heparan sulfate and chondroitin sulfate caused a dose-dependent inhibition of the RANTES-mediated infectivity enhancement (Fig. 6a). In the absence of RANTES,

![Diagram](https://via.placeholder.com/150)

**FIG. 4.** RANTES and AOP-RANTES, but not BB-10520 RANTES, multimerize upon binding to heparin. Radiolabeled RANTES (.), AOP-RANTES (A), or BB-10520 RANTES (B) was incubated with heparin-Sepharose beads in the presence of increasing amounts of the same, unlabeled chemokine, and the amount of radiolabeled chemokine bound to the beads was determined. The values shown are the means (± standard deviations) from five independent experiments.

![Diagram](https://via.placeholder.com/150)

**FIG. 5.** RANTES-mediated infectivity enhancement is dependent upon GAG expression on target cells. CHO-K1 cells (.), heparan sulfate-deficient pgsD-677 cells (.), or GAG-deficient pgsA-745 cells (A) were infected with HIV-1\(_{VSV}\) (1.5 ng of HIV-1 p24 antigen) in the presence or absence of the indicated concentrations of RANTES. Unbound virus was removed after a 2-h incubation, and the cultures were replenished with fresh medium without RANTES. (a) RANTES was added to the cells for 24 h, and then the chemokine-containing medium was washed away immediately before the addition of virus. RANTES was absent during the 2-h infection period and subsequently. (b) RANTES was added during the 2-h infection period but was not present prior to or after that time. The extent of viral infection was measured by determination of luciferase expression in quadruplicate cultures on day 3 postinfection; the data (mean ± standard deviation) are presented as percentages of control (no RANTES = 100%). The untreated control values (in RLU) were as follows: (a) CHO-K1 cells, 71.4 ± 16.3; pgsD 677 cells, 19.3 ± 4.4; pgsA 745 cells, 71.8 ± 8.4; (b) CHO-K1 cells, 47.4 ± 11.5; pgsD 677 cells, 22.9 ± 6.9; pgsA 745 cells, 37.8 ± 4.0.)
neither soluble GAG affected viral infectivity (Fig. 6a). Similar results were obtained when HIV-1 HXB2 (Env pseudotype) was substituted for HIV-1 MuLV (data not shown). When RANTES and soluble GAGs were both present during the period of virus-cell attachment and infection, the GAGs again reversed the enhancing effect of RANTES in a dose-dependent manner (Fig. 6b). However, the interpretation of this result is complicated by the inhibition of viral infectivity caused by GAGs in the absence of RANTES, a phenomenon that has been described previously (39, 63). Of note is the fact that soluble chondroitin sulfate inhibits the attachment of HIV-1 MuLV virions to RANTES-coated magnetic beads (Fig. 3a).

Effect of signal transduction inhibitors on RANTES-mediated infectivity enhancement. We have noted previously the correlation between the concentrations of RANTES that enhance viral infectivity and those that were reported by Bacon et al. to cause a large, sustained increase in cytosolic Ca\(^{2+}\) concentrations in CD4\(^+\) T cells (6, 7, 25). This increase in intracellular Ca\(^{2+}\) was sensitive to the protein tyrosine kinase inhibitor herbimycin A, whereas smaller, more transient Ca\(^{2+}\) increases induced by lower concentrations of RANTES were blocked by pertussis toxin, an inhibitor of signaling via G-protein-coupled receptors (6, 7, 25). We therefore tested whether the RANTES-induced enhancements of viral infectivity were affected by herbimycin A (Fig. 7).

The enhancement of HIV-1 MuLV infectivity caused by RANTES pretreatment of HeLa-CD4 cells was inhibited by herbimycin A in a dose-dependent manner (Fig. 7a). In the absence of RANTES, herbimycin A had no significant effect on HIV-1 MuLV infectivity (Fig. 7a). Similar results were obtained when herbimycin A was added to the target cells 48 instead of 25 h prior to infection (data not shown). In contrast, herbimycin A pretreatment for 25 h had no effect on the infectivity enhancement which occurred when RANTES was added to the HeLa-CD4 cells simultaneously with HIV-1 MuLV (Fig. 7b). To exclude the possibility that herbimycin A was no longer active after it had been in contact with the cells for 25 h, we repeated this experiment but with only a 1-h interval between the addition of herbimycin A and HIV-1 MuLV; the results were identical (data not shown). Under the conditions used in both Fig. 7a and b, the same pattern of data was obtained when HIV-1 HxB2 (Env pseudotype) was substituted for HIV-1 MuLV (data not shown).
not shown). Thus, the identity of the viral envelope glycoproteins which mediate entry into the target cells does not influence the RANTES-induced infectivity enhancement mechanisms or their sensitivity to herbimycin A.

**RANTES increases the efficiency of cell-cell fusion.** To gain more insight into the effects of treating target cells with RANTES for prolonged periods, we tested whether such cells were more permissive to cell-cell fusion and not just virus-cell fusion. To do this, we used an assay in which a luciferase reporter gene is transactivated when cell-cell fusion occurs (28, 61). HeLa cells expressing the envelope glycoproteins of HIV-1IIIB (HeLa-EnvIIIB cells) are the effector cells, and luciferase-containing HeLa-CD4 cells are the targets.

RANTES, at 5 to 10 μg/ml, caused an increase in the extent of cell-cell fusion, both when it was added to the mixed effector and target cell population only during the fusion reaction and when it was added to the effector cells for 24 h prior to the initiation of cell-cell fusion (Fig. 8). In contrast, the same concentrations of MIP-1α had little or no effect on cell-cell fusion under these conditions (Fig. 8). Thus, whatever changes are caused to HeLa cells by prolonged exposure to RANTES, their effect is to increase the extent of both virus-cell fusion and Env-mediated cell-cell fusion.

**DISCUSSION**

The purpose of this study was to understand the mechanisms by which the CC-chemokine RANTES enhances the infectivities of HIV-1 and other enveloped viruses when present at concentrations in excess of 500 ng/ml in vitro (36). We do not argue that what we have observed is necessarily physiologically relevant—plasma concentrations of RANTES rarely exceed 200 ng/ml in HIV-1-infected or uninfected people (42, 48, 52, 60)—although we note that local concentrations of RANTES in tissues are unknown but could be rather higher than in plasma, especially at the sites of inflammation, where RANTES performs its normal physiological functions. Neither do we expect that plasma concentrations of exogenously administered RANTES or its derivatives would approach the range at which viral infectivity enhancement occurs, if and when these compounds are used therapeutically. It should not be overlooked that concentrations of RANTES lower than those we have studied here can inhibit the replication of R5 HIV-1 isolates in vitro by preventing the use of the CCR5 coreceptor by these viruses (5, 10, 22, 29, 51, 64, 80, 85).

We believe, however, that understanding how RANTES enhances viral infectivity in vitro might throw light on the fundamental processes of viral infection, in particular for HIV-1. The complexity of the phenomena described here could also help explain the various, seemingly contradictory reports that RANTES can either inhibit or enhance HIV-1 replication in primary monocytes/macrophages (1, 3, 12, 29, 44, 59, 78, 96). We have not yet studied these cells in detail, but we and others have noted that RANTES can either inhibit or enhance the replication of X4 and R5X4 HIV-1 isolates in primary CD4+ T cells in a donor-dependent manner (26, 45, 58, 83, 85). We are presently investigating this phenomenon, to see whether it is mechanistically related to what we have observed here and previously (36).

In the present study, our principal findings are twofold: firstly, that oligomers of RANTES can cross-link enveloped viruses, including HIV-1, to cells via GAGs that are present on the membranes of both virions and cells; secondly, that oligomers of RANTES form on cell surface GAGs and transduce a herbimycin A-sensitive signal which, over a period of several hours, renders the cells more permissive to infection by HIV-1 or envelope pseudotypes of HIV-1. These phenomena may be relevant to studies of several viruses, because we have observed the first process with HIV-1, HIV-1Mol.V, and HIV-1VSV and the second with HIV-1, HIV-1Mol.V, HIV-1VSV, and influenza and vaccinia viruses (36, 83) (see above).

The observation that RANTES oligomers can cross-link viruses to cells via GAGs is consistent with previous studies emphasizing the importance of GAGs in virus-cell binding. Mondor et al. reported that the attachment of the TCLA strain of HIV-1tnt to HeLa-CD4 cells was strongly dependent on the binding of the virus to cell surface GAGs (heparans); attach-
ment could be efficiently inhibited by soluble heparin, dextran sulfate, or pentosan polysulfate but not by chondroitin sulfate (54). The importance of virus-GAG interactions, mediated by envelope glycoproteins or other virion components, for HIV-1 infection of various cell lines is well characterized (62, 71, 77); indeed, the rate-limiting step in HIV-1 penetration of its target cells is attachment to the cell surface, not the subsequent fusion reaction (68). This is also true of many other viruses (18, 57, 72, 90), and as with HIV-1, interactions with cell surface GAGs can be used to increase virion infectivity. For example, GAGs play an important role in facilitating interactions of herpes simplex virus with its fusion receptors (55, 95) and of the following viruses with the target cell surface: Dengue virus (16), vaccinia virus (19), foot-and-mouth disease virus (40), Sindbis virus (46), human herpesvirus 7 (79), and pseudorabies virus (86).

Virus-cell attachment can also be mediated by virion-associated adhesion factors binding to cell surface counterreceptors (11, 33, 67, 76) or, as we show here, by oligomers of RANTES. The mechanism and route by which virus-cell fusion subsequently occurs are irrelevant. We have found that RANTES oligomers can enhance the infectivity of VSV and MuLV Env pseudotypes of HIV-1, which enter target cells independently of CD4 and coreceptors (36). Similarly, the infectivities of murine influenza virus and vaccinia virus are also enhanced by oligomerized RANTES (83). The subsequent association of viruses with specific fusion receptors (e.g., of HIV-1 with CD4 and coreceptors) is still necessary for fusion and infection to occur. But an elevated concentration of virions attached to the cell surface increases the rate of virus-cell fusion, however fusion is achieved and also however attachment occurs.

In addition to virus-cell attachment, GAGs play an important role in the binding of other proteins to the cell surface. Thus, the major surface protein of the malaria sporozoites, the circumsporozoite protein, contains a highly conserved sequence responsible for the specific homing of malaria sporozoites to hepatocytes, the target cells for the first stage of infection. Their ability to oligomerize and cause infectivity enhancement lies on residues in this region interferes with the binding of this chemokine with GAGs (10). Of note is the fact that a single-residue change (glutamic acid to serine) in BB-10520 RANTES that abolishes its ability to oligomerize and cause infectivity enhancement lies at position 66, only two amino acids away from the C terminus (34).

The GAG-binding region of RANTES is also positively charged and is located close to its C terminus. Thus, Burns et al. found that lysine and arginine residues within the C-terminal α-helical region of RANTES are critical for the interaction of this chemokine with GAGs (10). Of note is the fact that a monoclonal antibody (4A12) whose epitope is heavily dependent on residues in this region interferes with the binding of RANTES to cell surface GAGs; this process is necessary for the transduction of Ca2+ signals and the antiviral action of low concentrations of RANTES (10). The single-residue change (glutamic acid to serine) in BB-10520 RANTES that abolishes its ability to oligomerize and cause infectivity enhancement lies at position 66, only two amino acids away from the C terminus and within the 4A12 epitope (10, 24).

The three-dimensional structures of chemokines clearly show that these molecules can dimerize in a manner that is not GAG dependent. The dimeric topology of CC-chemokines, as illustrated by RANTES (20), differs considerably from that of CXC-chemokines, for example, interleukin-8 (21). Furthermore, the crystal structure of MCP-1 reveals that this CC-chemokine can also form tetramers (50), yet MCP-1 binds to its seven-transmembrane receptor as a monomer (70). Whether it is monomeric or higher-order forms of RANTES that bind, at low nanomolar concentrations, to high-affinity receptors such as CCR1 and CCR5 (6, 75, 80) remains to be determined. It should be noted that while the E66→S mutation abrogates the ability of RANTES to form multimers, the protein is still able to dimerize (Fig. 4) (24). Dimerization is clearly not sufficient for a CC-chemokine to cause viral infectivity enhancement; MIP-1α is another molecule which dimerizes yet does not enhance infectivity (36, 37). One difference between RANTES and MIP-1α is that, at physiological pH, the former is cationic and the latter is anionic (20). Yet charge cannot be the sole determinant of whether a CC-chemokine can increase viral infectivity; MCP-1 is also cationic and oligomerizes upon binding to heparin (50), but it does not increase HIV-1 infectivity (36, 37). Clearly, aspects of the chemistry and structure of RANTES that influence its ability to cause viral infectivity enhancement remain to be identified.

We do not yet understand how the prolonged interactions of RANTES oligomers with cell surface GAGs render target cells more permissive for infection by HIV-1 and other enveloped viruses. We did, however, note the correlation between the concentrations of RANTES that enhance viral infectivity (36) and those found by Bacon et al. to cause a large, sustained increase in cytosolic Ca2+ concentrations in CD4+ T cells (6, 7, 25). Since this increase in intracellular Ca2+ was sensitive to the protein tyrosine kinase inhibitor herbimycin A (6, 7, 25), we tested the effect of this compound on the RANTES-induced viral infectivity enhancement and found that the enhancement was also herbimycin A sensitive. Dairagh et al. suggested that intracellular Ca2+ increases stimulated by high concentrations of RANTES were associated with CD3 expression (25). Our experiments, however, were all performed on CD3-negative cells, so the expression of CD3 is not necessary for infectivity enhancement to occur.

We suggest, therefore, that the binding of RANTES oligomers to cell-surface GAGs activates a signal transduction pathway(s) which involves herbimycin A-sensitive tyrosine kinases. Several signaling pathways activated by RANTES in T cells have been described (6, 7, 25, 43, 82, 87, 91, 93). At present, those involved in viral infectivity enhancement remain to be identified, as does the stage(s) in the life cycles of HIV-1 and other viruses that is affected by this signal(s). Syndecans are one group of prototypic proteoglycans that have been extensively studied; their expression changes dramatically during cell development and differentiation and is influenced by cell activation (13). Syndecans and another proteoglycan, CD44, have been shown to associate with protein tyrosine kinases from the Src family (38, 69). Useful information might accrue from studies in these general areas.

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