Detection of tryptase TL_2 and CD26 antigen in brain-derived cells non-permissive to T-cell line-tropic human immunodeficiency virus type 1

Nobuaki Shimizu[a], Mitsuru Kobayashi[a], Hui-Yu Liu[a], Hiroshi Kido[b], Hiroo Hoshino[a,*]

[a]*Department of Hygiene and Virology, Gunma University School of Medicine, Showa-machi, Maebashi, Gunma 371, Japan*
[b]*Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan*

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Abstract Tryptase TL_2 purified from MOLT-4 human T cells binds to the envelope protein of human immunodeficiency virus type 1 (HIV-1). Tryptase TL_2 and CD26 antigen are supposed to play roles in HIV-1 entry into cells. Although CD4 is a principal receptor for HIV-1, brain cells expressing the CD4 antigen are not permissive to HIV-1 strains infectious to monocyte or T-cell lines. We examined whether the non-permissiveness of the brain-derived cells to standard HIV-1 strains could be explained by a lack of tryptase TL_2 or CD26. Western blots showed that the amounts of tryptase TL_2 expressed in cell lysates prepared from the brain-derived cells were similar to those prepared from various cells susceptible to HIV-1 strains. Furthermore, flow cytometry revealed the presence of the CD26 antigen on the cell surface of many types of cells. The resistance of the brain-derived cells to standard HIV-1 strains is not due to a lack of tryptase TL_2 or CD26.

Key words: HIV-1; Tryptase TL_2; CD26; Brain cell; Tropism

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS [1–3]. Numerous HIV-1 strains have been isolated by co-cultivation of peripheral blood lymphocytes (PBL) of HIV-1 positive subjects with PHA-stimulated PBL or human T-cell lines [2–4]. HIV-1 strains infectious to human T-cell lines such as MOLT-4, MT-4, MT-2, CEM, H9 or C8166, or monocytic cell lines such as U937, have been preferentially isolated from patients who show clinical signs of AIDS or AIDS-related complex [3–6]. These isolates, however, will not enter cells derived from the human brain or skin, even though these cells are made to express the CD4 antigen [7,8]. It has also been reported that mouse cells expressing the human CD4 antigen are not infectable with standard HIV-1 strains [9]. These lines of evidence suggested that the presence of the CD4 antigen is not sufficient to support HIV-1 entry into cells.

Tryptase TL_2 has been reported to bind to the gp120 envelope protein and it is supposed to play a role in HIV-1 entry into cells [10,11]. This enzyme is a serine protease expressed on the cell surface of the MOLT-4 human T-cell line. The antiserum against it inhibits syncytium formation induced by the co-cultivation of MOLT-4 cells with HIV-1 producing cells. We suggested that the binding of the V3 loop of gp120 to tryptase TL_2 is necessary for infection with HIV-1 [11]. This enzyme recognizes the glycine–proline–glycine–arginine (GPGR) sequence, which is also located at the tip of the V3 loop of most standard HIV-1 strains.

We reported the isolation of HIV-1 variants infectious to CD4-positive brain cells from a T-cell line-tropic, wild-type virus [12]. This variant has a glycine–serine–glycine–arginine (GSGR) sequence at the top of the V3 loop. The wild-type viruses which have the GPGR sequence in the V3 loop readily infect T-cell line cells, but hardly infect brain-derived cells even though they express CD4. Thus, brain-derived cells may lack tryptase TL_2. We examined whether tryptase TL_2 is present in brain-derived cells and T cells by Western immunoblotting.

Recently, the CD26 antigen has been reported as a possible cofactor in HIV-1 infection and it may be responsible for determining the cell tropism of HIV-1 [13]. There seems to be controversy among reports concerning the role of CD26 in HIV-1 infection [14–18]. We also examined its expression in brain-derived cells.

2. Materials and methods

2.1. Cells and viruses

The human T-cell lines MT-4 [19], MOLT-4 [20] and C8166 [21] were maintained in RPMI1640 medium supplemented with 10% (v/v) fetal calf serum (FCS). Human glioma cell lines U-87 MG [22], U-251 MG [23], NP-1 and NP-2 [24], and U-87/CD4 stably expressing the human CD4 gene [7] were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% FCS. The human cervical carcinoma cell line HeLa [25], the CD4-expressing subline HeLa/CD4 [26] and the human osteosarcoma cell line HOS [27] were also maintained in EMEM with 10% FCS. The human fibroblast-like cell strains BT-2 and BT-3 derived from surgically dissected human astrocytoma and meningioma, respectively, were cultured as described [12]. The expression of CD4 mRNA and CD4 antigen in BT-2 and BT-3 cells was confirmed by Northern blotting and immunoprecipitation (unpublished data). Human peripheral blood lymphocytes (PBL) were isolated by Ficoll-Paque (Pharmacia, Uppsala) gradient centrifugation, stimulated with PHA-P (1:1000) for 2 days, then cultured in RPMI1640 medium containing 10% FCS and 200 units/ml recombinant interleukin 2.

The HIV-1 strains GUN-1/WT and GUN-1/V were isolated as described elsewhere [12,28]. Briefly, GUN-1 was isolated from a Japanese hemophiliac by co-cultivating his PBL with MT-4 cells. The GUN-1/WT substrain 5M was obtained by transfecting infectious GUN-1/rWT DNA into MT-4 cells. The GUN-1/V substrain r9V was isolated by adapting GUN-1/rWT virus (5M) to U-87/CD4 cells. Culture fluids of MOLT-4 cells persistently infected with GUN-1/WT and HTLV-III_B strains [4] and U-87/CD4 cells infected with GUN-1/V were used as the sources of HIV-1 strains.

2.2. Virus infection

Adherent cells were seeded into 12 well-plates at a density of $3–5 \times 10^4$ cells in 2 ml of culture medium. On the following day, the medium was removed and serially diluted viruses were inoculated onto them. The lowest dilutions of inocula were adjusted so as to contain 4.0×10^5 cpm of reverse transcriptase (RT) activity. 2 h later, the inocula were removed, then the cells were passaged every 5–6 days. Non-adherent MOLT-4, C8166 or MT-4 cells (1×10^5) were incubated with viruses for

*Corresponding author. Fax: (81) (272) 20-8006.

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2 h, washed once and cultured for 4 days. The percentages of cells expressing HIV-1 antigens were determined by indirect immunofluorescence assay (IFA) as described elsewhere [29].

2.3. Western blot

Western blotting proceeded as described previously [10]. Briefly, cells were suspended in lysis buffer consisting of 50 mM Tris-HCl (pH 6.5), 0.2% 3-[(3-chloamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.5 M NaCl, 50 μM L-*trans*-epoxysuccinyl-leucylamido-(4-guanidinobutane) (E-64), 50 μM chymostatin, 50 μM leupeptin, 50 μM 1,10-phenanthroline and 50 μM bestatin and homogenized. After centrifugation of the cell lysates at 25,000 × *g* for 20 min, the supernatants were used as samples. The cell lysates containing 5 μg protein were resolved by 10–20% polyacrylamide gradient gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and transferred to nitrocellulose membranes. These were incubated with polyclonal anti-tryptase TL_2 rabbit antiserum and examined using the pico Blue Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Bands detected were scanned with a densitometer (Atto, Tokyo).

2.4. Flow cytometry of the CD26 and CD4 antigens on the cell surface

Adherent cells were incubated with phosphate-buffered saline (PBS) containing 0.5 mM EDTA for 10 min at 37°C, then mechanically detached using a cell scraper. Cell suspensions (1×10^7 cells/ml) were incubated with the Ta1 anti-CD26 (Coulter Immunology, Hialeah, FL) or Leu3a (Becton Dickinson, San Jose, CA) or NU TH/I (Nichirei, Tokyo, Japan) anti-CD4 monoclonal antibodies at 0°C for 20 min. Then cells were incubated with fluorescent isocyanate-conjugated rabbit anti-mouse IgG as the second antibody at 0°C for 20 min, then fixed with 1% paraformaldehyde. In some experiments, cells were first treated with 10% normal rabbit serum in PBS to block Fc receptors on the cell surface and processed as described above. The expression of the CD26 or CD4 antigens on the surface of various cells was examined using a flow cytometer (CytoACE 150; JASCO Co., Tokyo). The ratios of antigen-positive cells were determined as described below. Cells stained with the second antibody alone provided the negative control. The cut-off level of fluorescence intensity of each cell line, by which 95 and 5% of the control cells were classified as being stained negative and positive, respectively, was determined as shown by the arrows in Fig. 3. Ratios of antigen-positive cells (%) were determined after incubating cells with the first and second antibodies as follows: percentage of antigen-positive cells = percentage of cells with a fluorescence intensity that was over a cut-off value of 5%.

3. Results

3.1. Susceptibility of human cells to HIV-1

We compared the susceptibility of human T cells and brain-derived cells to HIV-1. The human T-cell lines C8166, MT-4 and MOLT-4 were infected with three HIV-1 strains and examined by IFA after cultivation for 4 days. MT-4 and C8166 cells were readily infected with the $HTLV\text{-}III_B$, GUN-1/WT or GUN-1/V strains of HIV-1 (Fig. 1A and data not shown). Although a low ratio of MOLT-4 cells were positive for HIV-1 antigens after infections with the three viruses (Fig. 1B), almost all cells became positive for HIV-1 antigens after several passages (data not shown). HeLa/CD4 cells were also susceptible to $THLV\text{-}III_B$ (Fig. 1C) and most cells became infected after a few cell passages (data not shown). GUN-1/WT plated much less efficiently onto HeLa/CD4 cells than $HTLV\text{-}III_B$ (Fig. 1C): most cells still became positive for HIV-1 antigens after several passages. In contrast, GUN-1/WT or $HTLV\text{-}III_B$ could not plate onto brain-derived BT-2, BT-3 or U-87/CD4 cells (Fig. 1D–F). Even after several cell passages, these cells remained negative for HIV-1 antigens (< 1%). That is, human T cells or HeLa/CD4 cells were infectable with both types of HIV-1 strains and permitted further spread of HIV-1 to uninfected cells. In contrast, CD4-positive, brain-derived cells were almost completely resistant to standard T-cell line-tropic strains, and the secondary spread of HIV-1 in them was not detected even after many cell passages. The GUN-1/V strain plated efficiently onto these brain-derived cells (Fig. 1D–F). All cell lines which did not express CD4 antigen, such as U-87 MG, U-251 MG, NP-1, NP-2, HeLa and HOS cells (Table 1), were not susceptible to the three HIV-1 strains. Thus the CD4 antigen was necessary for the infection of these cells with HIV-1.

3.2. Western blots of tryptase TL_2

Brain-derived cells were resistant to infection with standard HIV-1 strains as reported [7,8]. We examined whether tryptase

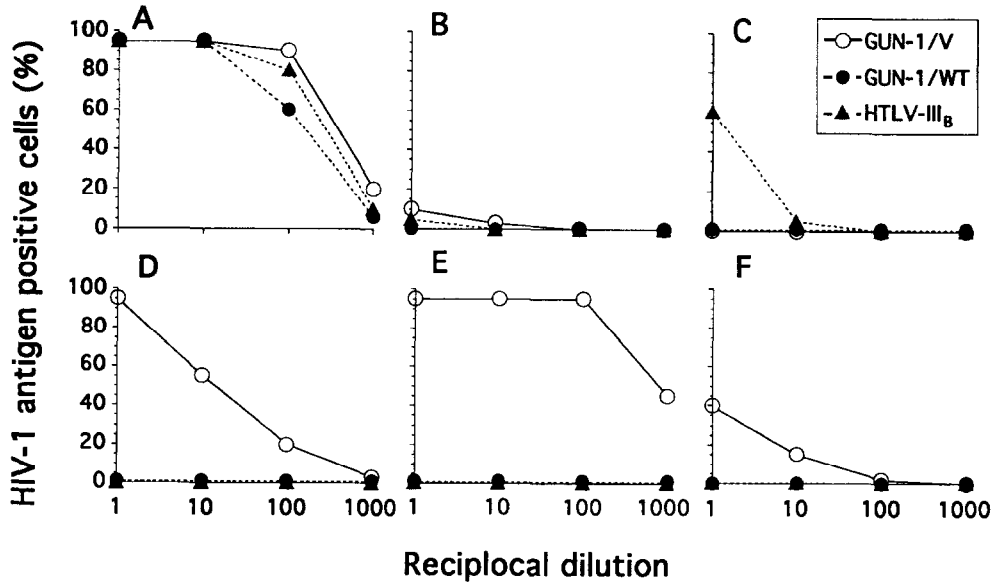

Fig. 1. The susceptibility of human cells to three HIV-1 strains. Cells were infected with HIV-1 and the expression of HIV-1 antigens was detected by IFA. (A) MT-4; (B) MOLT-4; (C) HeLa/CD4; (D) BT-2; (E) BT-3; (F) U-87/CD4.

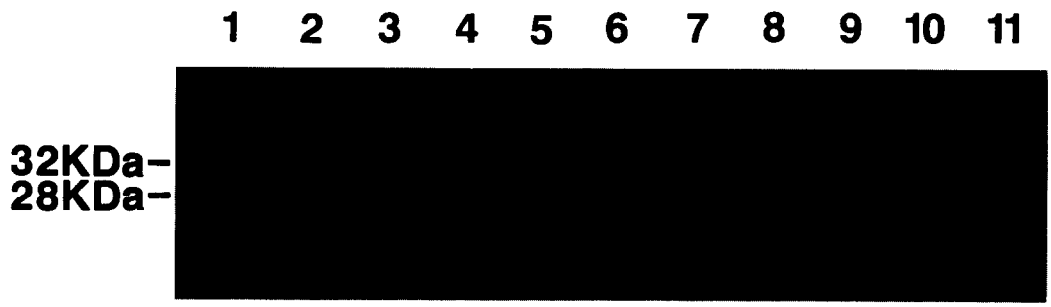

Fig. 2. Western blots of tryptase TL_2 in various human cells. Cell lysates were resolved by SDS. Lanes: 1, MOLT-4; 2, U-251 MG; 3, NP-1; 4, NP-2; 5, U-87 MG; 6, U-87/CD4; 7, BT-2; 8, BT-3; 9, HOS; 10, HeLa; 11, HeLa/CD4.

TL_2 was present in brain-derived cells as well as human T cells. A polyclonal antibody against tryptase TL_2 was used for Western blotting. In all the cells examined, we detected two bands corresponding to apparent molecular masses of 32 and 28 kDa (Fig. 2), which were considered to be the two main subunits of tryptase TL_2, as reported [10], although amounts of tryptase TL_2 expressed on the cell surface were unknown. The bands form MOLT-4 cells were the most prominent, and the relative intensities of other bands were determined by densitometry (Table 1). The BT-2 cell lysate gave the faintest bands and the relative intensities of the two bands of the other cells were within 20–57% of those of MOLT-4 cells. Although the amounts of tryptase TL_2 expressed in U-87/CD4, MT-4 and HeLa/CD4 cells were similar, there was a marked difference in their susceptibility to standard HIV-1 strains.

3.3. Flow cytometry of CD26 and CD4 antigen

The CD26 antigen was another potential cofactor of HIV-1 infection [13]. The expression of the CD26 or CD4 antigens on the cell surface was examined by flow cytometry. When PBL were reacted with anti-CD4 monoclonal antibody, two peaks appeared (Fig. 3A). When other cells were examined, single peaks of fluorescence intensity shifted to the right if cells were positive for CD26 or CD4 antigen. About 90% of PHA-stimulated PBL expressed CD26 antigen (Fig. 3 and Table 1). Half of the MOLT-4 cells and about 20% of U-87/CD4, U-87 MG and HOS cells were judged to be positive for this antigen (Fig. 3 and Table 1). As for other cell lines such as C8166 or MT-4 cells, less than 10% of the cells were positive for the CD26 antigen on their cell surface. Brain-derived cells may express Fc receptors on their surface. However, about half of U-87 MG and U-87/CD4 cells were again positive for CD26 even when these cells had been treated with 10% normal rabbit serum before anti- CD26 monoclonal antibody in order to block Fc receptors (data not shown). The CD26 antigen was expressed on the cell surface of not only T cells, but also of brain-derived cells such as U-87/CD4 and U-87 MG.

Expression of CD4 antigen was examined to evaluate our assay conditions. All T-cell lines such as MOLT-4, C8166 or MT-4 and U-87/CD4 and HeLa/CD4 cells expressed CD4 antigen (Fig. 3 and Table 1) as reported [7,26,29,30]. About 15% of the BT-2 cells were positive for this antigen. Less than 3% of cells expressed CD4 antigen in other lines, and these cells were considered to be negative for CD4 antigen. Pretreatment of U-87 MG or U-87/CD4 cells with normal rabbit serum again did not essentially affect their ratios of CD4-positive cells (data not shown). The results for the CD4 antigen were consistent with those of previous reports.

Table 1
Detection of tryptase TL_2 and CD26 and CD4 antigens in various human cells

Cell		Tryptase TL_2 (%)		Antigen expression (%)	
Designation	Origin	32 kDa	28 kDa	CD26	CD4
MOLT-4	T cell leukemia	100[a]	59	48[b]	67
C8166	T cell of cord blood	ND[c]	ND	10	86
MT-4	T cell leukemia	ND	ND	8	85
U-251 MG	Glioblastoma	54	21	4	1
NP-1	Glioma	25	9.3	3	2
NP-2	Glioma	44	17	5	0
U-87 MG	Glioblastoma	53	13	68	0
U-87/CD4		49	20	53	76
BT-2	Astrocytoma	2.2	< 0.1	10	15
BT-3	Meningioma	32	18	NA[d]	NA
HOS	Osteosarcoma	57	12	19	3
HeLa	Cervical carcinoma	49	27	8	0
HeLa/CD4		47	17	5	19
PBL	Lymphocyte	ND	ND	94	25

[a] Relative intensities of bands determined by densitometry. The intensity of the 32 kDa band of MOLT-4 cells was taken as 100%.
[b] Antigen positive cells (%) were determined as described in section 2.
[c] ND, not done.
[d] NA, not applicable due to cell damage.

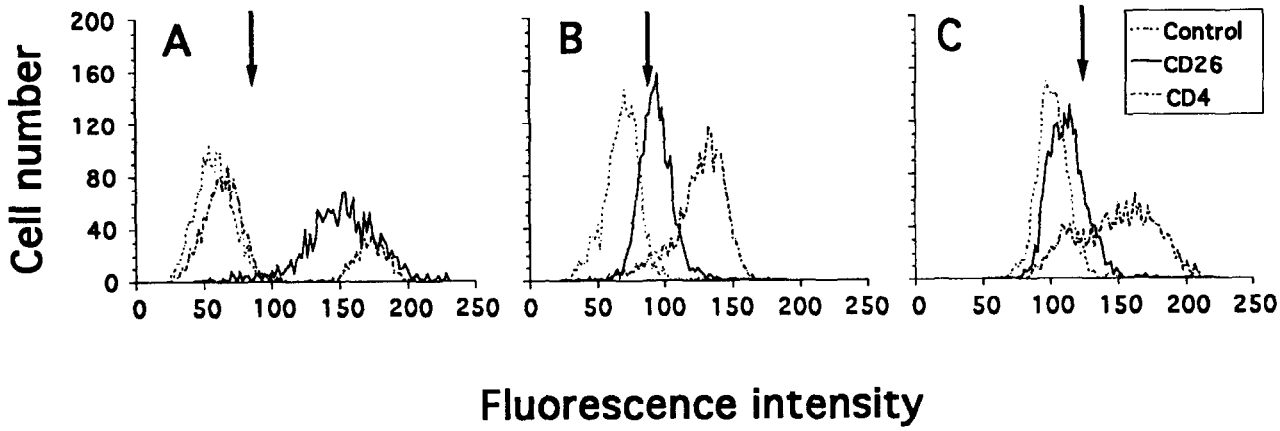

Fig. 3. Flow cytometry of the CD26 antigen on the cell surface. PBL (A), MOLT-4 (B) and U-87/CD4 (C) cells were reacted with Ta1 anti-CD26 monoclonal antibody and examined by flow cytometry. Arrows indicate the cut-off levels of the fluorescence intensity of the control cells which were reacted with the FITC-conjugated second antibody alone.

4. Discussion

During infection with paramyxovirus, such as Sendai virus or Newcastle disease virus, or retrovirus, such as HIV-1, viruses enter cells by means of membrane fusion, which occurs at the cell surface [31–33]. Other enveloped viruses, such as influenza virus, enter cells by endocytosis [34]. Cellular protease is thought to be necessary for the membrane fusion process: the fusion activity of envelope proteins will appear upon their proteolytic cleavage by host cell proteases [32,35,36]. Furthermore, the cell tropism of paramyxoviruses is reportedly determined by these proteases, while receptors for them are ubiquitously present on the cell surface [31,32]. The CD4 antigen serves as the main receptor for HIV-1, but an additional factor is supposed to be required for the entry of HIV-1 into cells. It is probable that the presence or absence of this factor is closely related with the cell tropism of HIV-1. Namely, the cell tropism of HIV-1 may be determined by a factor other than the CD4 antigen, one of which may be a cellular protease. If cells lack this factor or the substrate specificity of a putative protease in the cells is different from T cells, they may not be susceptible to standard HIV-1 strains.

Tryptase TL_2 is a serine protease that is located on the surface of the MOLT-4 human T-cell line. Its protease activity is markedly inhibited by compounds containing the glycine–proline–glycine–arginine (GPGR) or GPCR sequences such as Kuniz-type serine protease inhibitors, gp120 of HIV-1 or the oligopeptides derived from the V3 loop of gp120; furthermore, the GPGR sequence in the V3 loop is recognized by tryptase TL_2 [10].

T-cell line-tropic HIV-1 strains such as HTLV-III_B and GUN-1/WT that have the GPGR sequences at the tip of the V3 loop of gp120 plate well onto T-cell lines but not onto brain-derived, CD4-positive cells. We showed that a single point mutation at proline of the GPGR sequence in the V3 region is responsible for this brain cell tropism [12]. We further isolated new variants infectious to the brain-derived cells and they had a GSGR, GAGR or GTGR sequence at the tip of the V3 loop [37]. These findings suggested that brain cells may not recognize the GPGR sequence. In this report we confirmed that brain-derived cells were almost totally resistant to infection with T-cell line-tropic HIV-1 strains (Fig. 1) that have the GPGR sequence in the V3 loop.

Therefore, we examined whether the brain cells lack tryptase TL_2. Western blots showed that most cells expressed this protease whether they were derived from T cells, brain cells or other human tissues (Fig. 2). Tryptase TL_2 in MOLT-4 cells has recently been identified to be composed of at least three isomers with similar molecular masses and is localized not only in the cell membrane but also in the cytosol (Kido et al., in preparation). These data suggest that isoforms of tryptase TL_2 on the cell surface of brain-derived cells are different from those in T cell membranes, or that brain-derived cells may contain another protease which immunologically cross-reacts with anti-tryptase TL_2 antiserum but hardly reacts with the GPGR sequence. Furthermore, it remains to be investigated whether tryptase TL_2 isomers on the surface of T cells or brain-derived cells will recognize the GSGR, GAGR or GTGR sequence as well as the GPGR sequence. If the tryptase TL_2 isomers in brain-derived cells recognize the GPGR sequence, we should suppose that another factor determines the cell tropism of HIV-1.

The CD26 antigen has been implicated as a cofactor in the infection of T cells with HIV-1 [13]. This antigen has been described as dipeptidylpeptidase IV, which may recognize amino-terminal dipeptide motifs such as the GP, KP or RP sequence present in the V3 loop after endoproteolytic cleavage, and is abundantly expressed on the surface of activated T cells [13,38,39]. The possibility that brain cells lack this antigen was examined by flow cytometry (Fig. 3 and Table 1). These analyses showed that the human glioma cell lines, U-87 MG or U-87/CD4 expressed the CD26 antigen, as does the human T cell line, MOLT-4.

Tryptase TL_2 or CD26 antigen are thought to be cellular factors which are required for the entry of HIV-1 into cells and may also determine the cell tropism of HIV-1 strains. Here, we showed that the resistance of CD4-positive brain cells to standard HIV-1 strains could not be explained by a lack of tryptase TL_2 in the cells or CD26 antigen on the cell surface. It remains to be determined which factor is necessary for the entry of HIV-1 into cells in addition to the CD4 antigen.

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