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Original article

Construction and in vitro characterization of a chimeric simian and human immunodeficiency virus with the RANTES gene

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Abstract

Chimeric simian–human immunodeficiency virus (SHIV) containing the *env* gene of HIV-1 infects macaque monkeys and provides basic information that is useful for the development of HIV-1 vaccines. Regulated-on-activation-normal-T-cell-expressed-and-secreted (RANTES), a CC-chemokine, enhances antigen-specific T helper type-1 responses against HIV-1. With the final goal of testing the adjuvant effects of RANTES in SHIV-macaque models, we constructed a SHIV having the RANTES gene (SHIV-RANTES) and characterized its properties in vitro. SHIV-RANTES replicated both in human and monkey T cell lines. Along with SHIV-RANTES replication, RANTES was detected in the supernatant of human and monkey cell cultures, at maximal levels of 98.5 and 4.1 ng/ml, respectively. A flow cytometric analysis showed that the expressed RANTES down-modulated CC-chemokine receptor 5 (CCR5) on PM1 cells, which was restored by adding anti-RANTES antibody. UV-irradiated culture supernatants from the SHIV-RANTES-infected cells suppressed replication of CCR5-tropic HIV-1 BaL in PM-1 cells. Differentiating real-time RT-PCR showed that pre-infection of SHIV-RANTES in C8166 cells expressing CCR5 suppressed the replication of HIV-1 BaL. Biological activity of the expressed RANTES and the inserted RANTES gene in SHIV-RANTES remained stable after 10 passages. These results suggest that SHIV-RANTES is worth testing in macaque models.

Keywords: SHIV; RANTES; HIV-1; AIDS

1. Introduction

A successful HIV-1 vaccine is needed to control the worldwide AIDS epidemic. Chimeric simian and human immunodeficiency virus (SHIV) clones containing the HIV-1 *env* genes on a simian immunodeficiency virus (SIV) provide useful information on HIV-1 vaccine development, because SHIVs are readily infectious to macaque monkeys, and show induction of immune responses to HIV-1 Env. We previously reported the in vivo properties of the SHIV-NM3rN (derived from HIV-1 NL432 and SIV mac239) with deletion in the *vpx*, *vpr*, and/or *nef* genes [1]. Macaque monkeys inoculated with these gene-deleted SHIVs induced anti-HIV-1 Env humoral and cell-mediated immunity without causing an AIDS-like disease [1]. Moreover, the monkeys immunized with the *nef*-deleted SHIVs (SHIV-NI), were protected from a challenge with a heterologous pathogenic SHIV [2,3]. Live-attenuated SIV/SHIVs have been shown to be effective vaccines in macaque models. However, serious questions about the pathogenic potential of live-attenuated SIVs [4,5] have dampened enthusiasm for their use in clinical trials. Nevertheless, clarification of the protective mechanisms of attenuated SIV/SHIVs in macaque models could help to improve other vaccine candidates such as live vector-based vaccines and plasmid-DNA immunogens. In general, the immunogenicity of live-attenuated vaccines tends to increase with increasing virulence [6]. Therefore, in attenuating a live virus, there is a trade-off between safety and immunogenicity. A good way to overcome this problem is to genetically engi-

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neer a virus to co-express an immunostimulatory agent such as a cytokine adjuvant. Several studies have demonstrated that insertion of a cytokine in a gene-deleted live-attenuated SIV could boost its immunogenicity and enhance its protection ability [7,8]. This would make it possible to obtain a higher level of immunogenicity from safer, less virulent strains.

Chemokines constitute a family of small proinflammatory cytokines that regulate the activation and migration of leukocytes. Regulated-on-activation-normal-T-cell-expressed-and-secreted (RANTES) is a CC-chemokine and a natural ligand for the CC-chemokine receptors 1 (CCR1), CCR3, and CCR5. Receptors of RANTES are expressed on a variety of cells predominantly associated with T helper type-1 (Th1) responses [9]. An immune response polarized toward a more Th1 response is associated with a reduced viral load and non-progression of disease in HIV-1 infection. RANTES has been found to enhance cellular immune responses resulting in a more effective immune-modulating effect against HIV-1-related virus in rodent and monkey models [10–13]. In addition, infection of macaques with a live-attenuated SIV induced the production of CC-chemokines [14–16], and the up-regulation of CC-chemokines was found to be associated with the sterilizing immunity generated by the vaccine [14]. Moreover, RANTES has been shown to directly inhibit HIV-1 replication in vitro [17,18]. RANTES blocks or down-modulates CCR5 in vitro, which leads to suppression of CCR5-tropic (R5-tropic) HIV-1 infections. These results make RANTES an attractive candidate as a cytokine adjuvant.

To study the adjuvant effect of RANTES against HIV-1 related-virus infections in the macaque model, we have genetically engineered a SHIV to express the human RANTES gene (SHIV-RANTES). In this study, we compare the in vitro properties of SHIV-RANTES with those of its parental SHIV-NI. SHIV-RANTES replicates in both human and monkey cells, and expresses a high amount of RANTES. The RANTES produced with SHIV-RANTES was biologically active as shown by its ability to down-modulate expression of CCR5 and to inhibit the R5-tropic HIV-1 BaL infection. Pre-inoculating cells with SHIV-RANTES more efficiently suppressed a challenge with R5-tropic HIV-1 BaL in vitro than did pre-inoculation with the parental SHIV-NI. These results suggest that SHIV-RANTES will be useful for understanding the effect of RANTES against HIV-1-related infections. These data are an initial step toward the assessment of SHIV-RANTES in vivo.

2. Materials and methods

2.1. Construction of SHIV-RANTES

The SHIV-*nef* vector, designated as SHIV-NI, was constructed from an infectious molecular clone of SHIV-NM3rN [19]. The *env* gene of the SHIV-NM3rN was derived from CXCR4-tropic (X4-tropic) HIV-1 NL432, whose replication

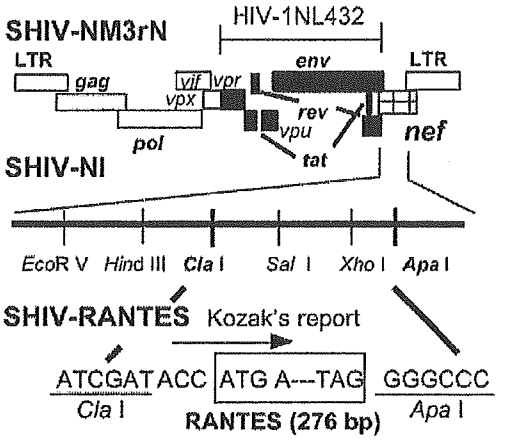

Fig. 1. Genetic structure of SHIV-RANTES. The *Cla* I and *Xho* I region of parental SHIV-NI, the *nef* cassette vector, was replaced by the human RANTES gene. The ORF including the initiation (ATG) and stop (TAG) codons of the RANTES gene is shown in the box. The flanking sequence of the RANTES initiation codon (ACCATGA) is an effective ribosomal initiation sequence based on Kozakn enzyme sites in place of the *nef* gene of SHIV-NM3rN. SHIV-NM3rN was constructed from HIV-1 NL432 's report (effective ribosomal initiation sequence: ANNATGN or GNNATGR). SHIV-NI has some unique restrictio(black regions) and SIV mac239 (white regions).

is not thought to be blocked by RANTES. In SHIV-NI, the *nef* gene was replaced by some unique restriction enzyme sites, including the *Cla* I and *Apa* I sites. The human RANTES open reading frame (ORF) was first amplified by PCR from a full length human RANTES cDNA as previously described [20]. The flanking sequence of the RANTES ORF was modified with the PCR primer RAN-Cla (5'-AT*ATCGAT*-ACCATGAAGGTCTCCGCGGCAG-3') and RAN-Apa (5'-TA*GGGCCC*CTAGCTCATCTCCAAAGAGTTG-3'). The underlined ATG in RAN-Cla indicates the start of the RANTES ORF. The relevant restriction sites in each primer are shown in italics. The flanking sequence of the RANTES initiation codon (ACCATGA) corresponds to an effective ribosomal initiation sequence based on Kozak's [21] report (effective ribosomal initiation sequence, ANNATGN or GNNATGR). The PCR product was cloned into pUC119 vector by TA-cloning, and the sequence was confirmed as previously described [22]. The PCR fragment digested with *Cla* I and *Apa* I was inserted into the SHIV-*nef* vector (Fig. 1).

2.2. Cell cultures

A $CD4^+$ human T lymphoid cell line, M8166 (a subclone of C8166), was used to prepare the stock virus and to measure the viral infectivity in human cells [23]. HSC-F cells, a cynomolgus monkey $CD4^+$ T cell line, were used to assess the viral infectivity in monkey cells [24]. PM1, a $CD4^+$ T cell clone that expresses CCR5, was derived from the human neoplastic T cells line Hut78 [25]. C8166-CCR5 cells were established by the transfection of C8166 cells with the human CCR5 coding region using retrovirus vector pMX-puro, which contains a puromycin-resistant gene [26]. PM1 and C8166-CCR5 were utilized as CCR5-expressing cells and R5-tropic

HIV-1-susceptible cells. These cell lines were all maintained in RPMI medium (RPMI 1640 with 2 mM L-glutamine and sodium bicarbonate; Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL Life Technologies, Auckland, New Zealand). Puromycin (1 μg/ml, Sigma) was added to the medium as a selection agent for C8166-CCR5 cells.

2.3. Virus stock

SHIV-RANTES and SHIV-NI was propagated as descried previously [22]. The virus stocks of the SHIV-RANTES and SHIV-NI were produced in M8166 cells, and the virion-associated reverse transcriptase (RT) activity of the virus stocks was measured. The titers of the viruses were determined by the 50% tissue culture infectious dose ($TCID_{50}$) method as described by Reed and Muench [27]. The $TCID_{50}$ of virus was correlated with the RT activity in this study. The laboratory monocytotropic HIV-1 BaL was utilized as R5-tropic HIV-1 [28]. The virus stock of HIV-1 BaL was prepared from the culture supernatants of HIV-1 BaL-infected PM1 cells.

2.4. Virus Infection of human and monkey cells

To investigate the kinetics of virus replication and production of RANTES with SHIV-RANTES in human cells, M8166 cells were inoculated with the virus as described elsewhere [22]. The virus inoculum was adjusted to contain a certain amount of RT units by adding the appropriate volume of the medium to the virus stock. Half of the culture supernatant was harvested with subsequent addition of new medium every 3 days. Virus replication kinetics was monitored by the RT activity of the supernatant. The production of RANTES from the virus-infected cells was measured by enzyme linked immunosorbent assay (ELISA) using a Quantikine human RANTES ELISA kit (R & D Systems, Inc., Minneapolis, MN). To assess the properties of SHIV-RANTES in monkey cells, HSC-F cells were also inoculated with the virus.

2.5. Flow cytometric analysis of CCR5 expression

RANTES down-modulates expression of CCR5 on the cell surface [29]. To assess the biological activity of the RANTES produced by virus-infected M8166 cells, the down-modulation of CCR5 in PM1 cells was evaluated. PM1 cells were plated at 2.5×10^5 cells per well, and incubated for 30 min at 37 °C with 100 μl of the culture supernatant of the samples. Thereafter, the cells were harvested and treated in staining buffer [phosphate buffered saline (PBS) containing 2% FCS and 0.1% sodium azide] for 20 min at 4 °C with phycoerythrin-conjugated anti-human CCR5 monoclonal antibodies (MAb) (2D7: PharMingen, San Diego, CA). To inactivate the virus, the cells were fixed in 4% paraformaldehyde for 30 min. Cells were analyzed for the cell surface expression of CCR5 by flow cytometry (EPICS XL ELITE: Beckman Coulter, Miami, FL). The percentage of CCR5 expression was calculated based on the samples without RANTES (0 ng/ml) which was defined as 100%. To assess the effect of the virus particles, the concentrations of the virions in the culture supernatants from the SHIV-RANTES-infected M8166 cells and SHIV-NI-infected cells were adjusted based on the virion associated-RT activity levels. To evaluate the effect of the spontaneous production of RANTES from the M8166 cells, the CCR5 expression on PM1 cells exposed to supernatants from virus-uninfected cells (hereafter referred to Mock) was also monitored. Serial dilutions of recombinant human RANTES (2–200 ng/ml; CHEMICON International, Inc., Temecula, CA) were used as controls. In the blocking assay with the anti-RANTES neutralizing antibodies, the supernatant samples were incubated with 25 μg/ml of MAb anti-human RANTES (R & D Systems) for 30 min at 37 °C before adding the samples to PM1 cells.

2.6. Inhibition of R5-tropic HIV-1 replication with the RANTES produced by SHIV-RANTES

To investigate whether the produced RANTES inhibits R5-tropic HIV-1 infections, the replication of HIV-1 BaL, an R5-tropic HIV-1, was monitored in the presence of the culture supernatants from the SHIV-RANTES-infected cells. The SHIVs in the culture supernatants were inactivated by UV-irradiation at 2 J/cm^2 to exclude the interference of replication of SHIVs on this assay. PM1 cells (5×10^4 cells per well) were incubated with the UV-irradiated samples, and infected with HIV-1 BaL. Half of each culture supernatant of PM1 cells was harvested with subsequent addition of new UV-irradiated samples every 3 days. The replication kinetics of HIV-1 BaL was monitored by the RT assay. To assess the influence of UV-irradiation, a recombinant human RANTES that had been exposed to a UV-source was also used.

2.7. In vitro challenge experiment

To assess whether the pre-inoculation with SHIV-RANTES inhibits R5-tropic HIV-1 replication, the SHIV-RANTES-infected C8166-CCR5 cells were challenged with HIV-1 BaL. C8166-CCR5 cells were pre-infected with SHIV-RANTES or SHIV-NI at 6 days before HIV-1 BaL infection. The inoculation of SHIV-RANTES and SHIV-NI were adjusted to the same RT levels. C8166-CCR5 cells were incubated for 2 h with SHIVs, washed two times with RPMI medium, and then cultured at 5×10^4 cells per well in a 96-well plate. Six days later, the SHIV-NI- and SHIV-RANTES-infected cells were co-infected with HIV-1 BaL. The culture supernatants of the C8166-CCR5 cells were harvested every 3 days. The growth kinetics of HIV-1 BaL and SHIVs was independently monitored with a differentiating real-time PCR quantification assay [2,30]. Total RNAs were prepared from the culture supernatants of virus-infected C8166-CCR5 cells with a QIAamp viral RNA kit (Qiagen,

Germany), and RT-PCR was performed using a TaqMan RT-PCR kit (Perkin–Elmer). The SIV *gag* region of the viral RNAs of SHIV-NI and SHIV-RANTES were amplified using the primers SIVII-696F (5'-GGAAATTACCCAGTACAA-CAAATAGG-3') and SIVII-784R (5'-TCTATCAATTTT-ACCCAAGGCATTTA-3'). A labeled probe SIVII-731T (5'-Fam-TGTCCACCTGCCATTAAGCCCG-Tamra-3') was used to quantify the PCR product. To detect RNA of the challenge virus HIV-1 BaL, nucleotide sequences for the HIV-1 *gag* region were amplified using the primers NL432-gag-F (5'-CAAGCAGCCATGCAAATGTTA-3') and NL432-gag-R (5'-GCATGCACTGGATGCAATCTAT-3'). A labeled probe NL432-gag-T (5'-Fam-AGAGACCATCAATGAGGAA-GCTGCAGAATG-Tamra-3') was added to the reaction mixture. These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and analyzed using the manufacturer's software. The viral RNA loads were quantified based on the copy number of the standard samples.

2.8. Stability of SHIV-RANTES after serial passages in vitro

SHIV-RANTES was inoculated to M8166 cells at 5×10^5 cells per well in a 24-well plate. When the cytopathic effect (CPE) was confluent, half of each culture supernatant was used to infect a well containing fresh M8166 cells. The culture supernatants from SHIV-RANTES-infected cells were passaged 10 times in this way. To analyze the inserted RANTES genes in SHIV-RANTES, the proviral DNA was amplified from the virus-infected cells after each passage and the length of the RANTES flanking region were checked by PCR [31].

3. Results

3.1. Replication of SHIV-RANTES and production of RANTES in human and monkey cells

In this study, a chimeric simian–human immunodeficiency virus having RANTES gene (SHIV-RANTES) was constructed (Fig. 1). SHIV-RANTES replicated well in human M8166 cells with almost the same replication competence as parental SHIV-NI (Fig. 2A). The RT activity of SHIV-RANTES peaked at about 9 days post infection (d.p.i.). The maximum level of RANTES in the culture supernatants was 98.5 ng/ml for the SHIV-RANTES-infected M8166 cells. The spontaneous production of RANTES was detected at levels between 4.1 and 9.3 ng/ml in the SHIV-NI-infected M8166 cells and the virus-uninfected M8166 cells control. Replication of SHIV-RANTES in monkey HSC-F cells, reached a peak at about 15 d.p.i. (Fig. 2B). The replication kinetics of SHIV-RANTES was similar to that of SHIV-NI. The maximum level of RANTES in the culture supernatants from SHIV-RANTES-infected HSC-F cells was at 4.1 ng/ml,

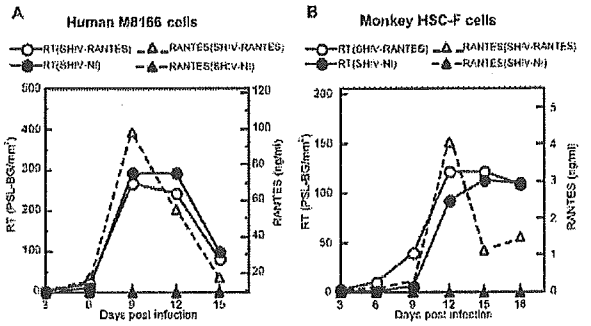

Fig. 2. Kinetics of virus replication and RANTES production with SHIV-RANTES and SHIV-NI in human (A) and monkey (B) $CD4^+$ cell lines. Viral replication was monitored by RT activity in the supernatant of cell cultures infected with SHIV-RANTES (open circles) or SHIV-NI (closed circles). The unit for RT activity is PSL-BG, photo-stimulated luminescence minus background (FLA-3000, Fuji Film, Japan). RANTES production (ng/ml) was detected by ELISA in the supernatant of cell cultures infected with SHIV-RANTES (open triangles) or SHIV-NI (closed triangles).

while it was less than the cut-off value (0.094 ng/ml) in the culture supernatants from the SHIV-NI-infected cells. SHIV-NI and SHIV-RANTES used CD4 and CXCR4, but not CCR5, as determined by an infection of GHOST cells expressing CXCR4 or CCR5 (data not shown). These data show that RANTES was produced efficiently along with the replication of SHIV-RANTES in human and monkey cells, and that the replications of SHIV-RANTES, X4-tropic virus, was not influenced by its production of RANTES.

3.2. Down-modulation of CCR5 by RANTES

RANTES down-modulates CCR5 from the cell surface, and CCR5 reaccumulates on the cell surface after the removal of ligands [29,32]. To check the biological activity of the produced RANTES, the down-modulation of CCR5 on PM1 cells was monitored. Surface expression of CCR5 was detectable on approximately 21% of PM1 cells with the anti-CCR5 MAb 2D7, which is consistent with a previous report [33]. In this study, the levels of RANTES in the cultures infected by SHIV-RANTES, SHIV-NI, and Mock were 14, 2.6, and 2.6 ng/ml, respectively. Treating the cells with recombinant human RANTES reduced expression of CCR5 on the cell surface in a dose-dependent manner (Fig. 3). When the PM1 cells were incubated with the culture supernatants from the SHIV-RANTES-infected cells, CCR5 expression on the cell surface was consistently reduced about 75%. Down-modulation of CCR5 was also observed in the cells treated with the culture supernatants from the SHIV-NI-infected cells, but the reduction was only about 33%, which was less than that in cells treated with culture supernatants from the SHIV-RANTES-infected cells. The down-modulation of CCR5 with the sample from the SHIV-NI-infected cells is thought to be due to the background concentration of RANTES, since the reduction was almost the same as that treated with the samples from the Mock (the down-modulation of CCR5 was 34%). Pretreatment of the samples with the anti-RANTES antibody

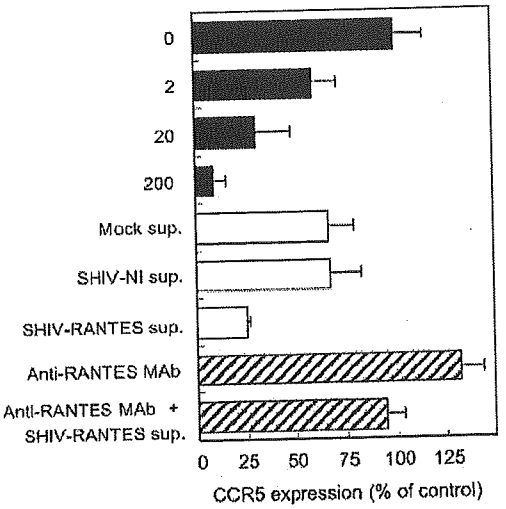

Fig. 3. Down-modulation of CCR5 on the surface of PM1 cells by RANTES. White bars, CCR5 expression on PM1 cells exposed to supernatant from cells infected with SHIV-RANTES, SHIV-NI, and uninfected cells (Mock). Black bars, PM1 cells treated with recombinant human RANTES at the indicated concentrations (in ng/ml). Hatched bars, cells treated with anti-RANTES antibody (Anti-RANTES MAb). These data are expressed as the mean of triplicate experiments ± S.E.M.

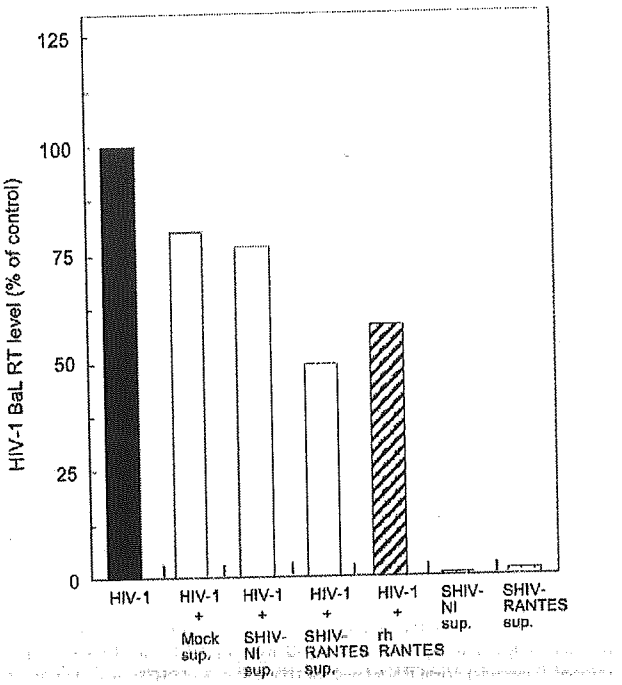

Fig. 4. Inhibition of HIV-1 BaL by UV-irradiated culture supernatants from SHIV-RANTES-infected cells. Virus replication of HIV-1 BaL was monitored by RT activity from the culture supernatants of virus-infected PM1 cells at 12 d.p.i. As control, cells treated with the same concentrations of UV-irradiated recombinant human RANTES were monitored (HIV-1 + rh RANTES). UV-irradiated supernatants from SHIV-RANTES and SHIV-NI-infected cells without HIV-1 BaL infection were used as negative controls. These data show one of three independent experiments with similar results.

completely abolished the down-modulation of CCR5. Thus, the reduction of the cell surface CCR5 correlates with the level of the expressed RANTES from SHIV-RANTES-inoculated cells.

3.3. Inhibition of R5-tropic HIV-1 BaL infection by RANTES from SHIV-RANTES-inoculated cells

In this study, the levels of RANTES in the UV-irradiated samples from the cultures infected with SHIV-RANTES, SHIV-NI, and Mock were 14, 0.8, and 0.8 ng/ml, respectively. In the case of the culture supernatants from SHIV-RANTES-infected cells, approximately 51% of the inhibition of HIV-1 BaL infection was consistently observed at 12 d.p.i., when the viral loads of the HIV-1 BaL peaked (Fig. 4). A reduction of the HIV-1 BaL RT activity was also observed for the culture supernatants from the SHIV-NI-inoculated cells and that of the Mock-inoculated cells, but the reductions were only about 23% and 20%, respectively. Interestingly, inhibition of HIV-1 BaL infection with the 14 ng/ml of the produced RANTES from the SHIV-RANTES-infected cells (51% inhibition) was slightly greater than inhibition with an equal concentration of recombinant human RANTES (42% inhibition). These results suggest that the produced RANTES efficiently inhibits R5-tropic HIV-1 infection.

3.4. In vitro challenge experiments of the SHIV-RANTES inoculated cells with R5-tropic HIV-1

A previous finding in the SIV-macaque models suggested that the induction of a CC-chemokine by a recombinant SIV vaccine was associated with protective immunity to mucosal infection with SIV [34]. We assessed whether the pre-inoculated cells with SHIV-RANTES were protected against the challenge with R5-tropic HIV-1 in in vitro experiments. In this assay, C8166-CCR5 cells were used as CCR5 positive cells, since the ability of virus replication in C8166-CCR5 cells was better than that in PM1 cells. As shown in Fig. 5A,B, the SHIV-RANTES-inoculated C8166-CCR5 cells produced RANTES, reaching a maximum level of 66.7 ng/ml at 6 days post challenge (d.p.c.) (at 12 d.p.i.). At the time of the challenge with HIV-1 BaL, the RANTES level in the culture supernatant of SHIV-RANTES-infected cells was already 16.9 ng/ml, while that of the SHIV-NI-infected cells was less than the cut-off value (2.5 ng/ml) during the experiment.

As shown in Fig. 5B, the viral loads of HIV-1 in the naive HIV-1 BaL-infected C8166-CCR5 cells increased to above 10^7 copies per ml at 9 d.p.c., and remained at a high level. Pre-inoculation of SHIV-RANTES suppressed the challenge of HIV-1 BaL. The peak virus loads of HIV-1 BaL in the SHIV-RANTES pre-inoculated C8166-CCR5 cells were two orders of magnitude lower than that of the naive HIV-1 BaL-inoculated cells. The viral loads of HIV-1 BaL in the culture supernatants of the SHIV-RANTES pre-inoculated cells plateaued at 10^4 copies per ml during the observation period. Viral loads of HIV-1 BaL in the SHIV-NI pre-infected cells reached between 10^5 and 10^6 copies per ml, which is higher

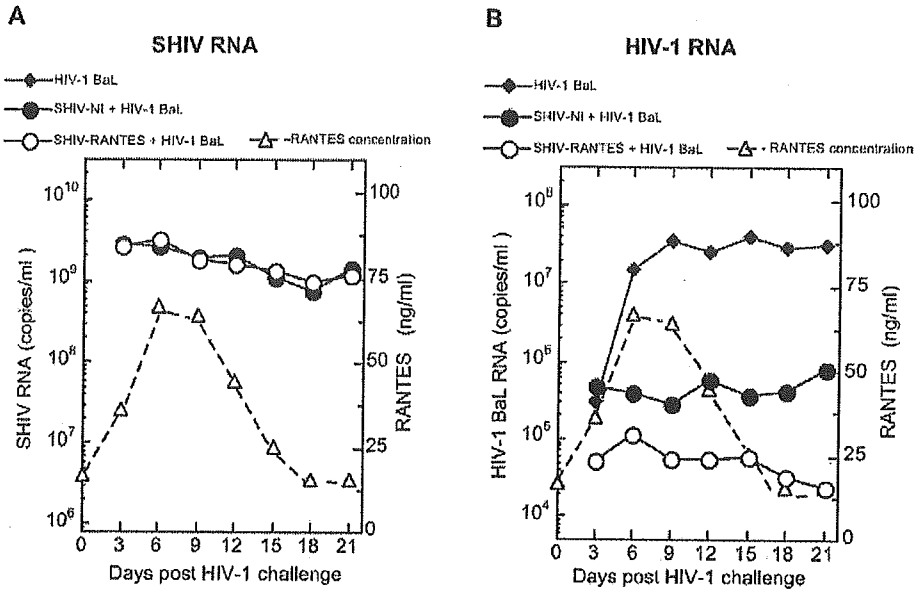

Fig. 5. Suppression of HIV-1 BaL by the pre-inoculation of SHIV-RANTES. Cells were pre-inoculated with SHIV-RANTES (open circles) and SHIV-NI (closed circles) at 6 days before the challenge of HIV-1 BaL. The HIV-1 BaL-infected cells without a pre-inoculation of SHIVs were used as a naive control (closed diamonds). Viral RNA levels of HIV-1 BaL and SHIVs were independently monitored by real-time RT-PCR using specific primer sets for either SIV *gag* region (A) or HIV *gag* region (B). Open triangles indicate RANTES levels produced by SHIV-RANTES, as determined by ELISA. (The RNA level of HIV-1 BaL detected by the primer for the SIV *gag* region was below 10^6 copies per ml).

than that in the SHIV-RANTES pre-inoculated cells. These results show that pre-inoculation of C8166-CCR5 with SHIV-RANTES partially protected against the challenge with HIV-1 BaL in vitro.

3.5. Stability of inserted RANTES gene in SHIV

Insertion of an additional gene may be a burden for the virus itself. In other studies, some cytokine genes such as IFN-γ, IL-2, and IL-12 inserted into either SHIV or SIV were found to be deleted during long-term experiments [19,35,36]. The stability of the inserted RANTES fragments in SHIV-RANTES after serial passage was examined by PCR of proviral DNA. These experiments were done more than three times. As shown in Fig. 6A, the inserted RANTES gene was found to be stable for at least 10 passages. The peaks of RANTES production in the culture supernatants from passage 1, 5, and 10 in the SHIV-RANTES-infected cells were 78.9, 76.8, and 82.2 ng/ml, respectively (Fig. 6B). These results show that SHIV-RANTES retained the ability to produce RANTES after serial passages. Furthermore, the expressed RANTES retained its ability to down-modulate CCR5 and to protect against HIV-1 BaL infection after 10 passages (Fig. 6C,D). These results show that the inserted RANTES gene in SHIV-RANTES was functional and stable until at least the 10th passage.

4. Discussion

In this paper, we compare the in vitro properties of SHIV-RANTES with those of its parental SHIV-NI. SIV/SHIV vectors containing a cytokine gene appear to be appropriate tools for observing the effect of local production of a cytokine on virus replication, pathogenesis, and immunogenicity, especially because the inserted cytokine gene is expressed in the region where the SIV/SHIV vector replicates. Because SIV dominantly utilized CCR5 for the virus entry and RANTES would suppress its replicate, we used the SHIV-NM3rN having the Env of X4-tropic HIV-1 NL432 as a vector that expresses RANTES genes. As expected, the replication of SHIV-RANTES, X4-tropic SHIV, was not suppressed by the expressed RANTES in this experiment.

SHIV-RANTES replicated well not only in the human $CD4^+$ T cell line M8166 but also in the monkey $CD4^+$ T cell line HSC-F. In addition, SHIV-RANTES successfully replicated in monkey PBMCs (data not shown). The expression level of RANTES in human M8166 cells was very high (up to 98.5 ng/ml). The high level of expression of RANTES may be partly due to the introduction of an effective ribosomal binding sequence at the flanking region of the RANTES ORF [21] and partly due to inserting the gene at the *nef* region [37]. We previously constructed the SHIV-*vpr* vector, named SHIV-3sj, from the same parental SHIV-NM3rN. Insertion of the RANTES gene into SHIV-3sj resulted in the expression of RANTES at a maximum level of 47.4 ng/ml in the culture supernatants of virus-infected M8166 cells [20]. The expression of RANTES with the SHIV-*nef* vector was about two times higher than that with the SHIV-*vpr* vector. A high level of expression of RANTES is advantageous to study the adjuvant effect of RANTES in vivo.

The results of many studies indicate that RANTES and its analogues suppress the infection of R5-tropic lentiviruses in

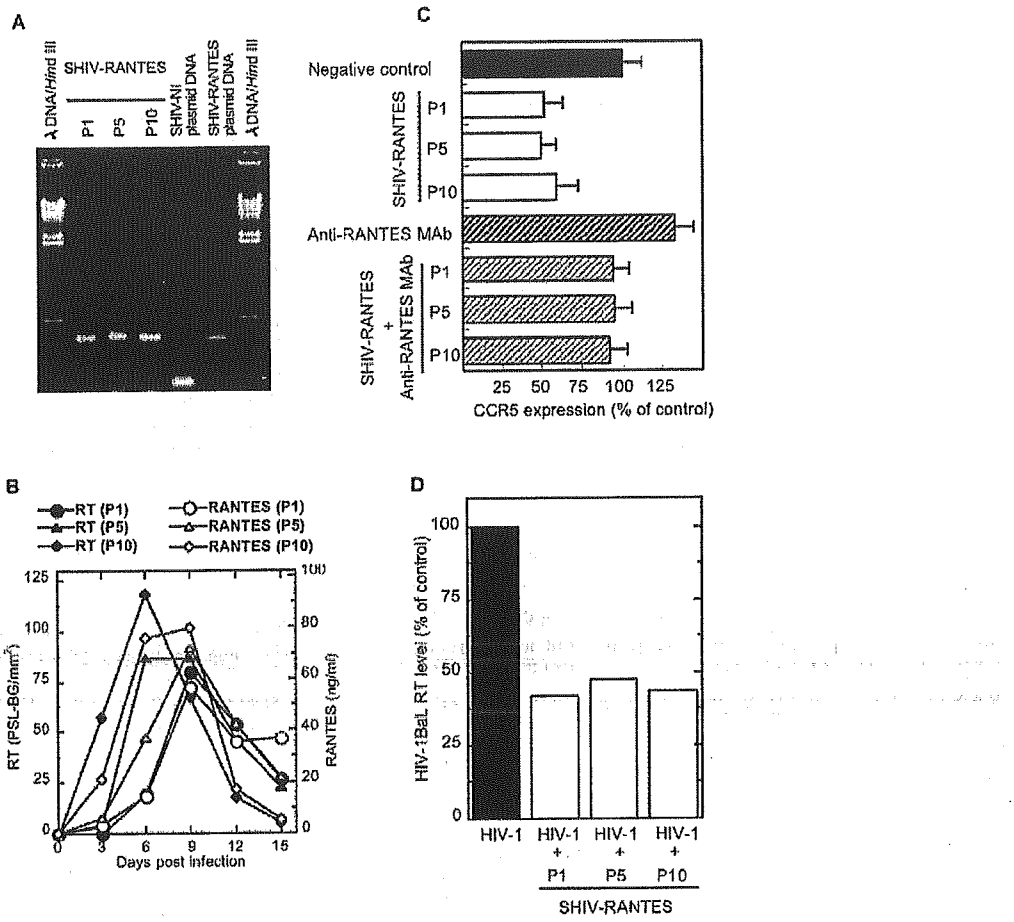

Fig. 6. Stability of SHIV-RANTES with serial passage. Culture supernatants from cells infected with SHIV-RANTES at passage 1 (P1), 5 (P5), and 10 (P10) were used for the experiments. (A) Stability of the inserted RANTES fragment in SHIV-RANTES was examined by PCR. DNA plasmids of SHIV-NI and SHIV-RANTES were used as the templates of the control. A λ DNA digested with *Hind* III was used as a size marker. (B) Kinetics of the RT activity and expression of RANTES at different passages. The unit for RT activity is PSL-BG, photo-stimulated luminescence minus background (FLA-3000, Fuji Film, Japan). The unit for RANTES (ng/ml) was determined by ELISA. (C) Down-modulation of CCR5 by RANTES expressed at different passages. The samples were also treated with anti-RANTES antibody to confirm the specificity of RANTES. (D) Inhibitory effect of R5-tropic HIV-1 BaL replication by RANTES produced by a serial passage of SHIV-RANTES.

vitro. However, little information is available on the role of RANTES on X4-tropic lentivirus replication. Previous studies reported that RANTES enhanced X4-tropic and R5X4-tropic HIV-1 replication, both of which depend on a signal transduction and the enhanced HIV-1 replication is associated with increased colocalization of CD4 and CXCR4 [38]. The over-expression of RANTES may increase the replication of X4-tropic SHIV. A safety concern about live-attenuated viruses is that their replication rate may increase. In vitro, however, we did not observe increased SHIV replication in either human or monkey cell lines infected with the RANTES-producing virus.

Although the goal of this study is to test the immune-modulating effect of RANTES against HIV-1-related virus infection, immune responses are difficult to assess in in vitro experiments. In vitro, the inhibitory effects of RANTES on the entry of R5-tropic strains of lentivirus are well established. RANTES blocks or down-modulates CCR5 in vitro, which suppresses HIV-1 infections [29,32]. The down-modulation of CCR5 is a biological activity of RANTES. Our findings that the produced RANTES down-modulated CCR5 expression on the cell surface of PM1 cells and that UV-treated samples from SHIV-RANTES-infected cells partially protected the PM1 cells from the R5-tropic HIV-1 BaL infection suggest that RANTES expressed by SHIV-RANTES-infected cells is the factor protecting against R5-tropic HIV-1 infection. In this study, the biological activity of RANTES was confirmed by its ability to down-modulate expression of CCR5 and to inhibit the R5-tropic HIV-1 BaL infection.

Our finding that the viral growth kinetics of HIV-1 BaL was more strongly inhibited by the RANTES produced by SHIV-RANTES-infected cells than by the recombinant RANTES may be due to the presence of inactivated virus particles and other inhibitory factors in the culture supernatants of the SHIV-RANTES-inoculated cells. Another reason

is that the RANTES produced by mammalian cells is structurally different from the recombinant RANTES. RANTES is secreted from some cells as a macromolecular complex containing sulfated proteoglycans (with molecular sizes of 400–600 kDa), whereas the molecular size of recombinant RANTES is approximately 7.8 kDa [39,40]. The interaction of chemokines with proteoglycans enhances their anti-HIV-1 activity [40,41]. In this experiment, after the separation into < 100 kDa fractions by ultrafiltration (UF) membranes, the concentration of the produced RANTES was reduced to 1.6% of the pre-filtered levels (from 39.3 to 0.61 ng/ml) (data not shown). Many studies have used recombinant RANTES to determine the role of RANTES in HIV-1 infection. In our SHIV-*nef* vector system, the RANTES is produced in a natural, secreted form, which may be an advantage for studying the effect of RANTES in vivo.

The immune responses induced by an attenuated virus can increase the immunity to a pathogenic virus. Compared with the immunostimulatory cytokines produced by other *nef* deletion mutants, the RANTES produced by SHIV-RANTES is expected to not only boost the primitive non-specific immunity and virus-specific immune response but also directly inhibit the entry of R5-tropic virus to susceptible cells. SHIV-RANTES has an ability to protect against challenge with a pathogenic virus when inoculated to monkeys. Indeed, we found that HIV-1 BaL replication was two orders of magnitude lower in the cells pre-inoculated with SHIV-RANTES than in the naive HIV-1 BaL-inoculated cells.

In conclusion, we have established a SHIV-*nef* vector system that stably expresses a high level of biologically active RANTES. Inoculation of macaque monkeys with SHIV-RANTES should provide further information on the immune-modulating effects of RANTES against lentiviral infections.

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Formation of Vesicular Stomatitis Virus Pseudotypes Bearing Surface Proteins of Hepatitis B Virus

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It has been difficult to propagate and titrate hepatitis B virus (HBV) in tissue culture. We examined whether vesicular stomatitis virus (VSV) pseudotypes bearing HBV surface (HBs) proteins infectious for human cell lines could be prepared. For this, expression plasmids for three surface proteins, L, M, and S, of HBV were made. 293T cells were then transfected with these plasmids either individually or in different combinations. 293T cells expressing HBs proteins were infected with VSVΔG*-G, a recombinant VSV expressing green fluorescent protein (GFP), to make VSV pseudotypes. Culture supernatants together with cells were harvested and sonicated for a short time. The infectivities of freshly harvested supernatants were determined by quantifying the number of cells expressing GFP after neutralization with anti-VSV serum and mouse monoclonal antibodies (MAbs) against HBs protein. Among 14 cell lines tested for susceptibility to HBV pseudotype samples, HepG2, JHH-7, and 293T cells were judged to be the most susceptible. Namely, the infectious units (IU) of the culture supernatant samples neutralized with anti-VSV in the absence and presence of anti-HBs S MAbs and titrated on HepG2 cells ranged from 1,000 to 4,000 IU/ml and 200 to 400 IU/ml, respectively, suggesting the presence of VSVΔG*(HBV) pseudotypes. This infectivity was inhibited by treatment with lactoferrin or dextran sulfate. Pretreatment of the cells with trypsin or tunicamycin inhibited plating of the pseudotype samples. The HBV pseudotypes can be used to analyze early steps of HBV infection, including the entry mechanism of HBV.

Hepatitis B virus (HBV), which belongs to the virus genus *Hepadnaviridae*, is an important etiological agent for acute and chronic hepatitis as well as liver cirrhosis or primary hepatocellular carcinoma (15, 18). The surface proteins of HBV contain three related HBV-encoded glycoprotein species, termed large (L), middle (M), and small (S) proteins (37). As shown in Fig. 1A, all surface proteins are produced from a single open reading frame (ORF) by the use of three different translational start sites that divide this ORF into three domains: preS1, preS2, and S. Translation from the first ATG yields L protein, comprising 390 amino acids (aa), whereas translation from the ATG at aa positions 109 and 164 generates M and S proteins, respectively. The three proteins are posttranslationally modified: each of them exhibits a glycosylation site in the S domain, and an additional glycosylation occurs in the preS2 region of the M protein (Fig. 1A). Either M or S protein, when expressed alone, can be secreted as virus-like particles (25, 45). In contrast, L protein cannot be secreted when expressed alone; instead, it is mainly retained within the endoplasmic reticulum and pre-Golgi regions in the form of intraluminal particles (27, 45). It has been reported that the sequence between amino acids 3 and 77 of the preS1 region is necessary for infection of primary human hepatocytes in vitro (21). The preS2 region of M protein has been thought to be involved in polymerized albumin-mediated interaction of HBV (48), but no precise function has yet been definitively assigned to the preS2 region of the M protein. Kann et al. (18) proposed that in association with the preS1 domain of L protein, the preS2 domain of M protein might have a supportive function in viral attachment. Thus, it is still necessary to study the roles of all three proteins in the HBV infection process to clarify the process of entry of HBV into cells.

Analysis of HBV infection in vitro has still been difficult due to the lack of a suitable culture system, i.e., lack of susceptible cell lines or of cells producing HBV abundantly. Most in vitro studies have used concentrated, whole virions or human serum samples as virus sources and primary human hepatocytes or human liver cell lines, e.g., HepG2, HuH7, and PH5CH8, as target cells (12, 13, 21, 31, 41). However, their infectivities are not so high, and human serum samples and human hepatocytes are not readily available. Despite these difficulties, infection of cells with viral inocula or transfection of cells with HBV DNA has been employed in order to analyze HBV infection in vitro (12, 14, 46). In vitro infection of human hepatoma (HepG2) cells with hepatitis B virus obtained from the serum of a chronic HBV patient was first reported by Ichini et al. (3).

There seems to be only one report that describes an HBV pseudotype: murine leukemia virus (MLV) pseudotype, expected to bear the L and S proteins of HBV (41). The reported MLV(HBV) pseudotype, which was prepared after cotransfection of 293T cells with plasmids expressing HBV surface (HBs) L and S proteins, infects only primary human hepatocytes, and up to 200 HBV antigen-positive cells were detected among 10^6 primary human hepatocytes inoculated with it.

Vesicular stomatitis virus (VSV) pseudotypes have been used to analyze early steps of infection with various human

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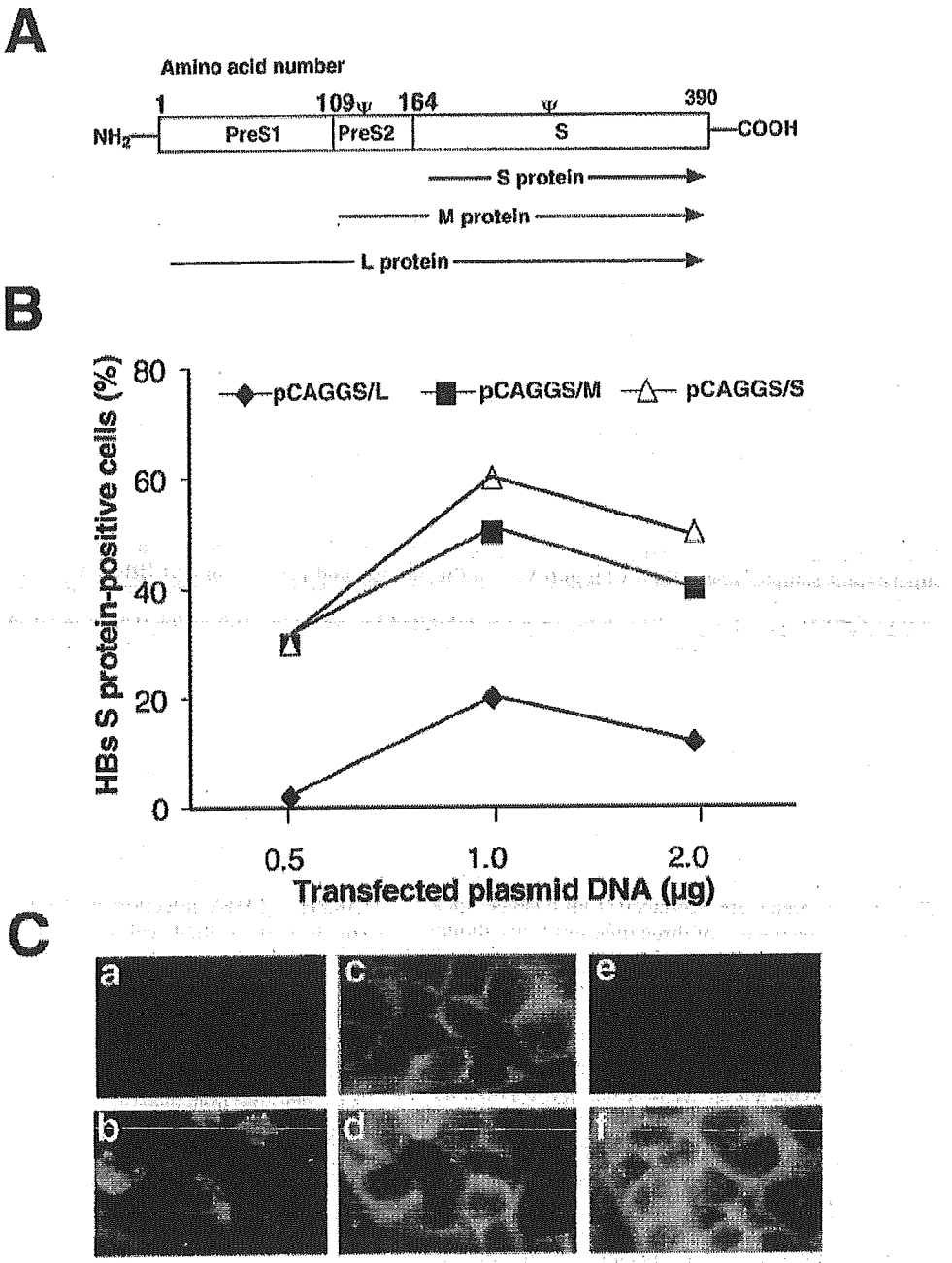

FIG. 1. Structure and expression of HBV surface proteins. (A) Schematic structure and expression of three surface proteins of HBV. Three surface antigens are expressed from a single ORF with three different translational start sites. The small protein (S) contains 226 amino acids, and the medium protein (M) and large protein (L) contain an additional 55 and 164 amino acids, respectively, in addition to the amino acid residues of the S protein. Glycosylation sites in the S and preS2 domain are represented by ψ symbols. (B) Ratios of cells expressing HBs proteins after transfection of HBs expression plasmid DNA. 293T cells were transfected with different concentrations of the expression plasmid containing the HBs L, M, or S gene, and the next day the cells were seeded onto chamber slides. The slides were fixed 1 day later with acetone and stained with mouse anti-HBs S MAb (DA-3E7). HBs protein-expressing 293T cells were detected by IFA. (C) Detection of HBV surface proteins in the transfected 293T cells and in Alexander cells by IFA. Expression of HBs in the transfected 293T cells was detected by using anti-HBs MAb, DA-3E7. 293T cells were transfected with pCAGGS (a), pCAGGS/L (b), pCAGGS/M (c), or pCAGGS/S (d) expression plasmids and examined by IFA. Alexander cells were seeded onto chamber slides, fixed with acetone 1 day later, and stained with mouse anti-HBs S MAb (CH-2D1.2). For a negative control, PBS(−) was used instead of anti-HBs MAb.

TABLE 1. Infectivity of the VSVΔG*(HBs/M) pseudotype sample to various human cell lines

Cell line	Origin	Infectious titer (IU/ml)[a]			
		VSVΔG*(HBs/M)[b]		VSVΔG* (−)[c]	VSVΔG*-G[d]
		(−)	(+)		
HepG2	Hepatoblastoma	3,800	400	400	6.0×10^8
JHH-7	Hepatocarcinoma	3,300	200	100	2.0×10^8
PH5CH8	Liver	200	100	100	1.2×10^8
Huh-7	Hepatoma	1,400	500	1,000	8.0×10^8
293T	Embryonic kidney	2,600	320	300	3.0×10^8
NP-2	Glioma	720	240	420	1.2×10^8
U251MG	Astrocytoma	520	240	320	9.2×10^8
HeLa	Cervical cancer	200	120	100	5.0×10^8
HOS	Osteosarcoma	160	100	100	2.0×10^7
C8166	T cells infected with HTLV-1	<20	<20	<20	3.6×10^8
C91/PL	T cells infected with HTLV-1	<20	<20	<20	3.0×10^8
HL60	Promyelocytic leukemia	<20	<20	40	2.0×10^7
MOLT4	T-cell acute lymphoblastic leukemia	<20	<20	<20	4.2×10^8
K562	Chronic myelogenous leukemia	60	<20	60	1.4×10^8

[a] Infectious titers were determined by counting the number of GFP-expressing cells 24 h after infection of the target cells.
[b] 293T cells were transfected with pCAGGS/M plasmid DNA and then infected with VSVΔG*-G to prepare the pseudotype sample. The VSVΔG*(HBs/M) sample was treated with anti-VSV in the absence (−) or presence (+) of anti-HBs S MAb (DA-3E7) and inoculated onto the target cells.
[c] Target cells were infected with the control samples prepared using 293T cells not transfected with pCAGGS/M plasmid DNA but infected with VSVΔG*-G.
[d] Target cells were infected with the VSVΔG*-G sample used for preparation of HBs pseudotype samples to examine their susceptibility to VSVΔG*-G.

viruses, like human T-cell leukemia virus type 1 (HTLV-1) (6), human immunodeficiency virus type 1 (HIV-1) (23), hepatitis C virus (HCV) (19), and Ebola virus (16). These pseudotypes will be especially useful for the study of viruses that do not grow well in tissue culture, like HCV or HTLV-1, or viruses that are highly biohazardous to humans, like Ebola virus. In addition, assays using the VSV pseudotypes can usually be done in a short time compared with those using the original viruses. In this study, we examined whether we could detect VSV pseudotypes bearing various surface proteins of HBV. We have been using VSVΔG*-G to prepare pseudotypes bearing surface proteins of HTLV-1 or HCV and found that the formation of these pseudotypes could be detected after treatment with highly active anti-VSV serum (43). Thus, using similar procedures to detect HTLV-1 and HCV pseudotypes, we tested whether VSV pseudotypes bearing HBs L, M, or S protein were formed and examined their reactivity with HBV-specific antibodies or chemicals. Results thus obtained showed that VSV pseudotypes bearing HBs proteins were formed.

MATERIALS AND METHODS

Cells. All cell lines used in this study are shown in Table 1. The 293T cell line was derived from the human embryonic kidney cell line 293 and contains the simian virus 40 large T antigen (9). The PH5CH8 cell line was cloned from nonneoplastic human hepatocytes, PH5CH cells (30). PH5CH8 cells were maintained as described previously (30). HepG2 cells were derived from human liver tumor biopsies (1). HepG2 and Huh-7 (26) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The JHH-7 cell line was derived from HBs antigen (HBsAg)-positive hepatocellular carcinoma with liver cirrhosis (10). PLC/PRF/5 cells (Alexander cells) were derived from a primary carcinoma of the liver of an HBsAg-positive patient (2). NP-2 (39), U251MG (4), and HeLa (36) cells were maintained in Eagle's minimum essential medium (Nissui) supplemented with 10% FBS. HL60, K562, MOLT4 (40), C8166 (35), MT-2, and C91/PL cells (33) were cultured in RPMI 1640 medium (Nissui) supplemented with 10% FBS. The K562 cell line was derived from Ph'-positive chronic myelogenous leukemia in BLAST crisis (22). The HL60 cell line was derived from promyelocytic leukemia (8). MT-2 is a T-cell line persistently infected with HTLV-1 (24).

Plasmids. The genes for three surface proteins of HBV, large (L), middle (M), and small (S), were amplified by PCR from the plasmid pBSadr4×2(R), which contains two copies of the full-length HBV genome (11), using the following sense primers:

L, 5′-TCGCGAATTCATGCAGTTAATCATTACTTC-3′; M, 5′-ATGCAG TGGAACTCCACAACATTCCACCAA-3′; and S, 5′-CACCGAATTCATGGA GAACACAACATCAGG-3′. As an antisense primer, 5′-GCAACCCCGATGA GGGAACTTAAGTACCCT-3′ was used. For the convenience of cloning manipulation, the L and S sense primers contained a flanking sequence with EcoRI sites, which are underlined above. The PCR products, using the L and S sense primers, were inserted into the expression plasmid pCAGGS (kindly provided by J. Miyazaki) (29). The PCR product, using the M sense primer, was first inserted into the TA cloning vector and then subcloned into pCAGGS. The sizes and orientations of the inserts in the cloned plasmid DNAs were confirmed by restriction enzyme digestion, and the inserts were subjected to partial sequencing of the plasmid DNAs. The pCAGGS/L, pCAGGS/M, and pCAGGS/S plasmids thus obtained were thereby confirmed to carry the genes for HBs L, M, and S proteins, respectively.

Virus. VSVΔG* is a recombinant VSV derived from a full-length cDNA clone of the VSV genome in which the coding region of the VSV G protein was replaced with that of the green fluorescent protein (GFP) gene (42). VSVΔG* pseudotype-bearing VSV G protein (VSVΔG*-G) was prepared by infecting 293T cells with VSVΔG*-G, which had been tranfected with the G protein expression plasmid DNA pCAGGS/VSV-G 1 day earlier, and culture supernatants were harvested after incubation for 24 h. VSVΔG*-G was kindly provided by M. A. Whitt.

Monoclonal antibodies. Anti-HBs monoclonal antibodies (MAbs) referred to in this paper stand for MAbs against S and preS2 domains of HBs. Different clones of anti-HBs S MAb, e.g., clone 3E7 (DAKO Japan Co., Ltd., Tokyo, Japan), clone ZCH16 (Zymed Laboratories, Inc.), clone 2D1.2 (Chemicon International Inc.), and anti-preS2 MAb (clone S26; Chemicon) were used in this study. In this text, these MAbs are designated DA-3E7, ZY-ZCH16, CH-2D1.2, and CH-S26. The antigenic determinants of all the anti-HBs S MAbs are supposed to locate in the "a" determinant region of the S domain that is specific for the subtype ad/ay. The antigenic determinant for the anti-preS2 MAb (CH-S26) is the 132- to 137-amino-acid region (adw subtype specific) of the preS2 domain. Due to the presence of the S domain in the L and M proteins, three anti-HBs S MAbs used in this experiment are expected to recognize all three surface proteins (7). Rat monoclonal antibody against HTLV-1, LAT-27, was a generous gift of Y. Tanaka of Rykyu University (44).

Expression of HBV surface proteins. The expression of HBV surface (HBs) proteins was detected by indirect immunofluorescense assay (IFA), flow cytometry (FCM), and Western blot (WB) analysis. Subconfluent 293T cells seeded in 6.0-cm plates were transfected with 0.5 to 2.0 μg of one of the L, M, or S expression plasmids or with a combination (total amount of DNA being 1.0 μg for each of these transfections) by using FuGENE 6.0 transfection reagent (Roche Pharmaceuticals, Mannheim, Germany) according to the protocol recommended by the manufacturer. For IFA, 1 day after DNA transfection the cells were detached from dishes and then seeded onto 14-well chamber slides to make cell smears for IFA. After overnight incubation at 37°C, the cells were fixed with acetone or 2.0% paraformaldehyde. Half of the paraformaldehyde-fixed cell samples were further treated with 0.1% Triton X-100 to make them permeabilized. Acetone- or paraformaldehyde-fixed smears of 293T cells were treated with

MAb against S protein (DA-3E7) at a 1:40 dilution for 1 h. After the smears were washed with phosphate-buffered saline (PBS) containing 0.1% sodium azide, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin G (IgG) (DAKO) diluted at 1:100 was added. After 1 h of incubation at 37°C, the cell smears were washed and examined under a fluorescence microscope. Expression of HBs in Alexander cells was determined by seeding the cells in 14-well chamber slides as described above and staining them with MAb against HBsAg (CH-2D1.2).

Flow cytometric analysis. 293T cells were detached from plates by pipetting and suspended in culture medium. These cells were then centrifuged, and the cell pellets were resuspended in PBS. The cells were fixed with 1.0% paraformaldehyde and then either permeabilized by treatment with 0.2% Saponin or left untreated. Permeabilized and nonpermeabilized cells were incubated separately with MAb against S protein (DA-3E7) diluted at 1:40 on ice for 1 h. After washing with wash buffer (PBS containing 1.0% FBS and 0.01% sodium azide), the cells were incubated on ice for 1 h with FITC-conjugated rabbit anti-mouse IgG (DAKO) diluted at 1:100. The cells were again washed with wash buffer and suspended in PBS containing 1.0% paraformaldehyde and finally analyzed using a CytoACE-150 flow cytometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan). Percentages of antigen-positive cells were determined as described previously (38).

Western blot analysis. 293T cells were transfected with the plasmids to express HBV surface antigen HBs S, M, or L protein. Forty-eight hours after transfection, the cells were collected, lysed with lysis buffer (25 mM Tris-Cl, pH 7.4, 250 mM Nacl, 5 mM EDTA, 1.0% NP-40), and centrifuged to collect cell lysates, and finally sample buffer (50 mM Tris-Cl, pH 6.8, 2.0% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol) with or without 100 mM dithiothreitol (DTT), abbreviated as DTT(+) or DTT(−), respectively, was added to the lysates. The samples were heated at 100°C for 5 min and subjected to SDS–12% polyacrylamide gel electrophoresis. Separated proteins in gels were blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, Mass.). HBV surface proteins were detected by staining the membranes with MAbs against S protein (DA-3E7, ZY-ZCH16, or CH-2D1.2) and with peroxidase-conjugated secondary antibody. Finally, an ECL kit (Amersham Biosciences Ltd., Buckinghamshire, United Kingdom) was used to detect bands in X-ray films. High-Range Rainbow Molecular Weight Markers (Amersham Biosciences) were used as size markers.

Preparation of pseudotype virus samples harboring HBs surface proteins. Culture plates used for transfection were coated with poly-L-lysine (PLL) (Sigma Aldrich Co. Ltd., St. Louis, Mo.). 293T cells (2.4×10^6 cells per 6.0-cm plate) were seeded into PLL-coated dishes, and the next day the cells were transfected with the HBs-expressing plasmid DNAs. The cells were infected 30 h later with VSVΔG*-G at a multiplicity of infection of 2.0. The virus was allowed to adsorb for 2.5 h at 37°C, and the cells were washed four times with DMEM without FBS and cultured in DMEM containing 2.0% FBS. After incubation for 21 h at 37°C, the cells were detached using cell scrapers (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and suspended in culture medium. The cell suspensions were sonicated with a Sonifier 250 (Branson Co., Ltd., Danbury, Conn.) for 1.0 s and centrifuged to collect the supernatants. The culture supernatants were then aliquoted and stored at −80°C. Collected VSV samples were designated VSVΔG*(HBs/L), VSVΔG*(HBs/M), and VSVΔG*(HBs/S), which were prepared using 293T cells transfected with pCAGGS/L, pCAGGS/M, and pCAGGS/S, respectively. VSVΔG*(HBs/L+M), VSVΔG*(HBs/M+S), VSVΔG*(HBs/L+S), and VSVΔG*(HBs/L+M+S) samples were similarly prepared after transfection of the two or three expression plasmids as explained below. Namely, pCAGGS/L+M indicates that a DNA mixture of pCAGGS/L and pCAGGS/M in equal amounts was transfected. In each combination, the total amount of DNA transfected was 1.0 μg. In addition, as a control, culture supernatant of the VSVΔG*(−) sample was harvested from mock-transfected 293T cells infected with VSVΔG*-G. VSVΔG*-G was also inoculated onto 293T cells transfected with HBs fragment-free pCAGGS, and the culture supernatant was harvested and used as a control virus, designated VSVΔG*(−). VSVΔG*-G was titrated using 293T cells.

Detection and neutralization of HBs pseudotypes. For detection of a VSV pseudotype bearing HBs proteins (HBs pseudotypes), target cells were seeded into 96-well plates. VSV samples (114 μl) prepared using 293T cells transfected with the HBs-expressing plasmids were mixed with goat anti-VSV serum (6 μl) and anti-HBs S MAbs (DA-3E7, ZY-ZCH16, and CH-2D1.2) or MAb against preS2 (CH-S26) at different dilutions and incubated for 30 min at 37°C. Fifty microliters of VSV mixture per well was then added onto the target cells in duplicate. The presence of the HBs pseudotypes was also tested by treating the VSV samples with commercially available hepatitis B immunoglobulin (HBIG) (Nippon Seiyaku Co., Ltd., Tokyo, Japan) prepared from pooled human anti-HBs antibody-positive sera. The mixture was then inoculated onto target cells in 96-well tissue culture plates. After adsorption for 2 h, the mixture was removed and the cells were washed by DMEM, which was further replaced with fresh medium. The cultures were incubated at 37°C, but HepG2 cells were incubated at 33°C after inoculation of the pseudotype samples. Cells expressing GFP were counted under a fluorescence microscope. In addition, a rat MAb against HTLV-1, LAT-27, was also used to check the specificity of the reaction of anti-HBs MAbs with the VSV pseudotypes.

Effect of bovine lactoferrin and sulfated polysaccharides on the infectivity of the pseudotypes. The VSV pseudotype sample was treated with various concentrations of bovine lactoferrin (bLF) (Sigma), heparin, dextran sulfate (molecular weight, 500,000), and dextran (molecular weight, 7,000) at 37°C for 1 h and then inoculated onto HepG2 cells. The number of GFP-positive cells was determined using a fluorescence microscope as described above.

Chemical modification of HepG2 cells. A glycosylation inhibitor, tunicamycin (Sigma), was added to the target HepG2 cells soon after seeding cells into 96-well plates, and the cells were incubated at 37°C overnight. HepG2 cells, seeded 1 day before infection, were treated with various concentrations of trypsin for 5 min, and an equal volume of fresh medium was added to the cells to stop the reaction. The pseudotype sample was inoculated onto these pretreated HepG2 cells, and the number of GFP-positive cells was determined as described above.

RESULTS

Detection of HBs surface proteins by IFA and FCM. The optimum level of expression of the surface proteins was determined by using different amounts of plasmid DNA for transfection. Staining of acetone-fixed cell smears with a MAb against HBs S protein (DA-3E7) by IFA showed that the expression levels of all three HBV surface (HBs) proteins were optimal at 1.0 μg of DNA (Fig. 1B). The intracellular expression after transfection with 1.0 μg of each HBs expression plasmid individually as detected by IFA is shown in Fig. 1C. HBs S or M protein was detected to be expressed at a similar efficiency with similar localization, while that of L was different. PLC/PRF/5 cells (Alexander cells) were stained positive with only one (CH-2D1.2) of the three MAbs tested. The localization of HBsAg in Alexander cells looked similar to that of 293T cells transfected with either S or M protein (Fig. 1C). JHH-7 cells did not stain positively with any of the anti-HBs MAbs tested (data not shown). In order to differentiate their intracellular expression from surface expression, transfected 293T cells were permeabilized with either Triton X-100 (for IFA) or Saponin (for FCM). About 5 to 10% of the cells were positive for L, M, and S proteins, whereas 10 to 30% of the cells treated with Triton X-100 were positive for L, M, and S proteins (Table 2). Flow cytometric (FCM) analysis results for HBs protein expression were also consistent with those of IFA. In FCM, about 10% of the cells were detected to express S and M proteins on the surface, but L protein was not detected on the surface at a significant level, whereas intracellular expression of L, M, and S proteins was detected in about 25 to 35% of the cells (Fig. 2A, Table 2).

Detection of HBs proteins by Western blot. 293T cells transfected with pCAGGS plasmids containing genes for HBs S, M, and L proteins were lysed, and their cell lysates were subjected to Western blot (WB) analysis in the presence (+) or absence (−) of DTT. The DTT(+) samples gave a positive reaction with none of HBs MAbs tested (data not shown). The DTT(−) samples gave positive reactions with two (CH-2D1.2 and ZY-ZCH16) of the three anti-HBs S MAbs tested. These results suggest that the antigenicity of the surface proteins detected by these MAbs is mostly conformation dependent in nature. Because of the presence of a glycosylation site in the S domain and an additional glycosylation site in the M domain (37), under reducing conditions, DTT(+), S, M, and L proteins could be detected as both major nonglycosylated and glycosylated forms of 24 and 27 kDa, 33 and 36 kDa, and 39 and 42

TABLE 2. Detection of HBV surface proteins by IFA and FCM

Transfected plasmid[a]	HBs S protein-positive cells (%) by:				
	IFA			FCM (paraformaldehyde fixed)	
	Acetone fixed	Paraformaldehyde fixed			
		Triton X-100[b]		Saponin[c]	
		(−)	(+)	(−)	(+)
pCAGGS	<0.2	<0.2	<0.2	1	1
pCAGGS/L	20	5	10	2	25
pCAGGS/M	50	10	30	12	33
pCAGGS/S	60	10	30	13	35

[a] 293T cells were transfected with one of the pCAGGS plasmids, and the transfected cells were analyzed by IFA or by FCM. HBs S-protein-positive cells were determined using anti-HBs S MAb (DA-3E7).

[b] Transfected cells were fixed with paraformaldehyde and then left untreated (−) or treated with (+) Triton X-100 for permeabilization of the cells.

[c] Transfected cells were detached from culture dishes, fixed with paraformaldehyde, and then left untreated (−) or treated with (+) 0.2% Saponin for permeabilization of the cells. The percent of HBs S-protein-positive cells was determined as described in Materials and Methods.

kDa, respectively, as reported by Lambert and Prange (20). Since we could detect WB bands only under nonreducing conditions, DTT(−), the bands corresponding to expected molecular sizes could not be detected. WB of the DTT(−) samples detected using MAb CH-2D1.2 (Fig. 2B) showed multiple bands, i.e., 33, 42, and 45 kDa in the lysates of cells transfected with pCAGGS/S. Transfection of pCAGGS/M plasmid gave bands with estimated sizes of 33, 42, 45, and 66 kDa, whereas the lysate of the pCAGGS/L-transfected cells gave two major bands of 45 and 88 kDa. After transfection of pCAGGS/M plasmid, several bands with similar sizes observed in the lane of pCAGGS/S were detected. Transfection of pCAGGS/L and pCAGGS/M plasmids gave L-plasmid-specific 88-kDa and M-plasmid-specific 66-kDa bands, respectively, whereas an S-plasmid-specific 45-kDa band was shared by all the transfected cell lysates, namely, cells transfected with pCAGGS/L, pCAGGS/M, or pCAGGS/S. The major bands of about 45, 66, and 88 kDa detected after transfection of 293T cells with pCAGGS/S, pCAGGS/M, and pCAGGS/L plasmids, respectively (Fig. 2B), might be explained by formation of dimer forms of the respective proteins. As shown in Fig. 2B, in addition to these major bands, other bands were also shown to be present in cases of transfection with plasmids containing genes of M and L proteins. These additional bands could be partly due to an internal initiation of translation from the translation site of the S protein. Even in cases of transfection with the HBs S expression plasmid, some of the additional bands were also detected (Fig. 2B, lane 1); partial deglycosylation might produce these bands.

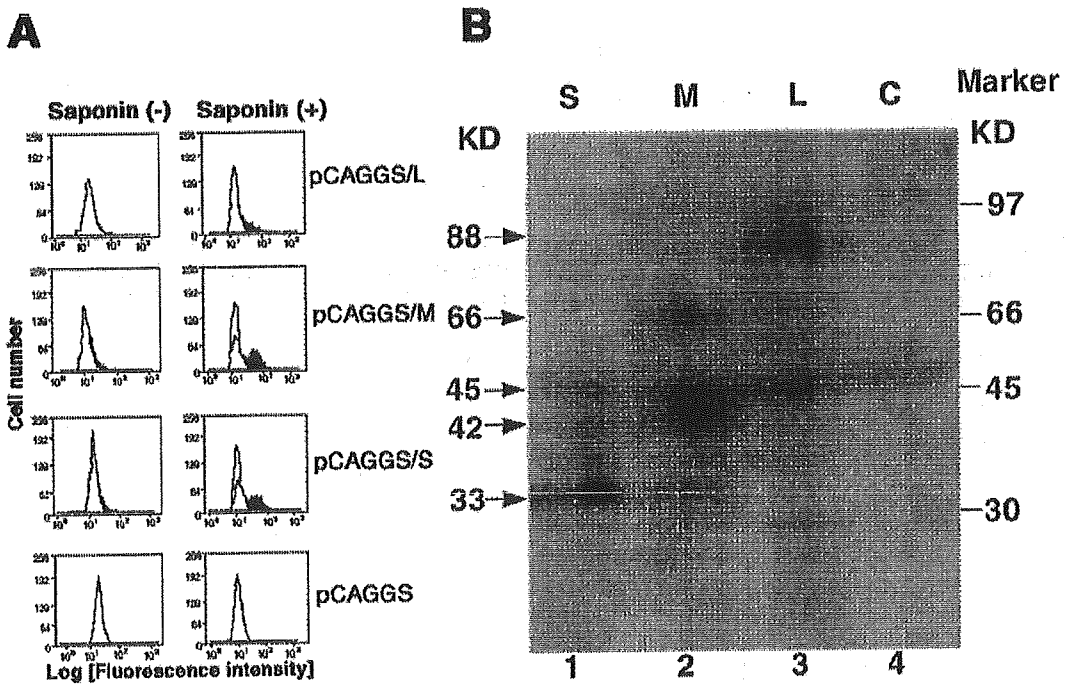

FIG. 2. Detection of HBs proteins. (A) FCM. For HBs expression, 293T cells were transfected with HBs expression plasmid DNAs, incubated for 48 h, and fixed with paraformaldehyde. The cells were then either permeabilized with 0.2% Saponin or left untreated and stained with mouse MAb (DA-3E7) against HBs S protein. Cells treated with the antibody were then incubated with FITC-conjugated rabbit anti-mouse IgG, and quantitative expression was measured in a flow cytometer. The background staining was provided by staining the cells with the FITC-conjugated secondary antibody alone (white areas). The specific staining with DA-3E7 MAb is shown by black areas. (B) Western blotting. 293T cells were transfected with the expression plasmid containing the HBs L, M, or S gene, and 48 h after transfection the cells were collected and lysed with lysis buffer. The lysates were mixed with sample buffer, subjected to SDS–12% polyacrylamide gel electrophoresis, and blotted onto a polyvinylidene difluoride membrane. The expression of HBs proteins was detected in samples prepared under nonreducing conditions with anti-HBs S MAb (CH-2D1.2). The expression of HBs proteins after transfection of pCAGGS/S, pCAGGS/M, and pCAGGS/L plasmids is shown in lanes 1, 2, and 3, respectively. The lysate of the cells transfected with the pCAGGS plasmid without an HBs gene insert was run in lane 4. The positions of the molecular size markers in kilodaltons (KD) are shown. Bands specifically detected by the MAb are indicated by arrows.

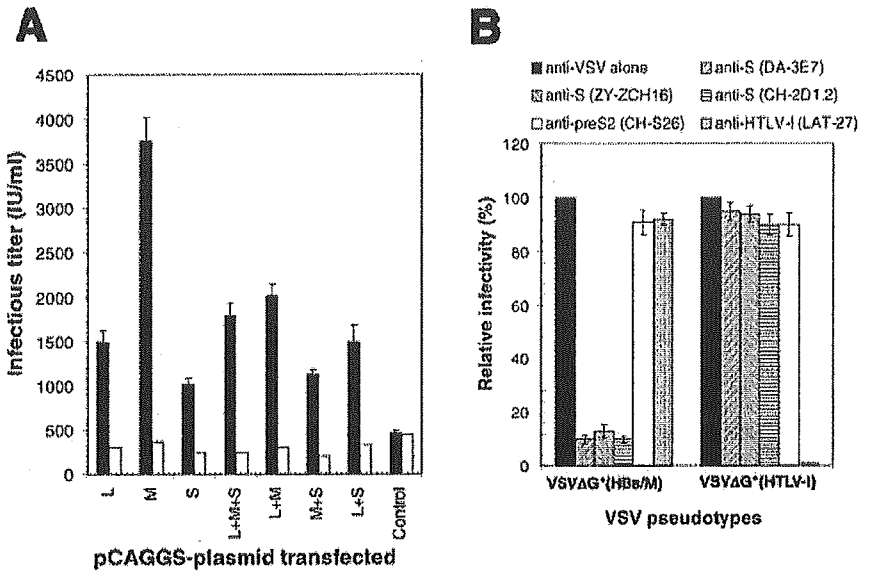

FIG. 3. Detection and neutralization of HBs pseudotypes. (A) Infectious titers of different pseudotype samples in HepG2 cells. 293T cells were transfected with one of the HBs expression plasmids or combinations of them. Namely, pCAGGS/L (L), pCAGGS/M (M), or pCAGGS/S (S) plasmid DNA or their mixture (L+M+S, L+M, M+S, or L+S) was transfected. After incubation for 32 h, the cells were infected with VSVΔG*-G, and 21 h after infection, HBs pseudotype samples were prepared after sonication. These samples were treated with goat anti-VSV serum in the absence (solid bar) or presence (open bar) of mouse MAb against HBs S protein (DA-3E7) for 30 min at 37°C, and 50 μl of them was inoculated onto HepG2 cells seeded into 96-well microplates 1 day before. After overnight incubation, the number of GFP-positive cells was counted under a fluorescence microscope. Each bar represents the means of infectious titer (infectious units per milliliter ± standard deviation) calculated from three independent experiments. (B) Neutralization of pseudotype activities by MAbs. VSVΔG*(HBs/M) and VSVΔG*(HTLV-1) samples were mixed with goat anti-VSV serum together with one of the MAbs (DA-3E7, ZY-ZCH16, and CH-2D1.2) against HBs S protein or anti-preS2 MAb (CH-S26) or a MAb against HTLV-1 (LAT-27). The antibody-treated pseudotype samples were inoculated onto HepG2 cells. After overnight incubation, the number of GFP-positive cells was counted under a fluorescence microscope. The experiments were done at least twice in duplicate, and the mean values ± standard deviations are shown.

Detection of VSVΔG*(HBV) pseudotypes. HepG2 cells, grown in 96-well plates, were used to detect formation of VSV pseudotypes. The culture samples prepared using 293T cells transfected with the HBs expression plasmids and infected with VSVΔG*-G were incubated with goat anti-VSV serum and anti-HBs S MAb (DA-3E7) and used to infect the HepG2 cells. Infectious units (IU/ml) of the VSVΔG*-G-infected samples were determined by counting the number of GFP-expressing cells. Candidate VSV pseudotype samples prepared by transfection of the plasmids coding for HBs L, M, and S proteins gave infectious titers of about 1,500, 3,800, and 1,000 IU/ml, respectively (Fig. 3A). Transfection of 293T cells with combinations of the expression plasmids did not further increase the infectivities. The infectivities of all these pseudotype samples were inhibited to the background level when treated with the anti-HBs S MAb, i.e., less than 300 IU/ml (Fig. 3A). The neutralization of the pseudotype infectivities by the anti-HBs S MAb suggests the successful formation of VSVΔG*(HBV) pseudotypes.

Neutralization of the pseudotype samples by MAbs. In order to confirm the presence of VSVΔG*(HBV) pseudotypes, neutralization tests with other MAbs against viral surface proteins were done. That is, 293T cells were transfected with pCAGGS/M plasmid and infected with VSVΔG*-G. The VSVΔG*-G-infected sample designated VSVΔG*(HBs/M) was prepared after sonication and tested for neutralization by the goat anti-VSV serum together with MAbs against HBV or HTLV-1. Figure 3B shows that the infectivity was markedly neutralized by the other two mouse anti-HBs S MAbs (ZY-ZCH16 and CH-2D1.2) as well. An anti-HTLV-1 MAb, LAT-27, did not affect its plating onto HepG2 cells, whereas a HTLV-1 pseudotype was well neutralized by LAT-27 but by none of the anti-HBs S MAbs (Fig. 3B). MAb against preS2 (CH-S26) neutralized neither VSVΔG*(HBs/M) nor VSVΔG*(HTLV-1) pseudotype sample. Thus, the infectivity of the VSVΔG*(HBs/M) sample was especially neutralized by the three HBs S protein-specific MAbs but not neutralized by anti-preS2 MAb or anti-HTLV-1 MAb (Fig. 3B). In addition, anti-HBs S MAb (DA-3E7) also neutralized the infectivity of VSVΔG*(HBs/L) and VSVΔG*(HBs/S) pseudotype samples (Fig. 3A), but anti-HTLV-1 MAb could neutralize none of these samples (data not shown).

Infectivity of the VSVΔG*(HBs/M) pseudotype sample for various cell lines. The VSVΔG*(HBs/M) sample prepared as described above was used to infect various target cells in order to study its cell tropism. Target cells were chosen according to the previous reports on their susceptibilities to HBV (13, 31, 41) and HCV (19, 43) infection. The only HBV pseudotype reported so far, MLV(HBV) pseudotype (41), exclusively infects only primary human hepatocytes but not any of the established cell lines tested, such as HepG2 or 293T. We were interested in examining whether any of the cell lines derived from human hepatoma, glioma, kidney, osteosarcoma, etc., could be infected by a VSVΔG*(HBs/M) pseudotype sample. Infectious titers of the VSVΔG*(HBs/M) samples in 14 different human cell lines were determined. As shown in Fig. 3 and Table 1, the VSVΔG*(HBs/M) sample was able to infect HepG2 cells. Among the other 13 cell lines, a hepatocarcinoma

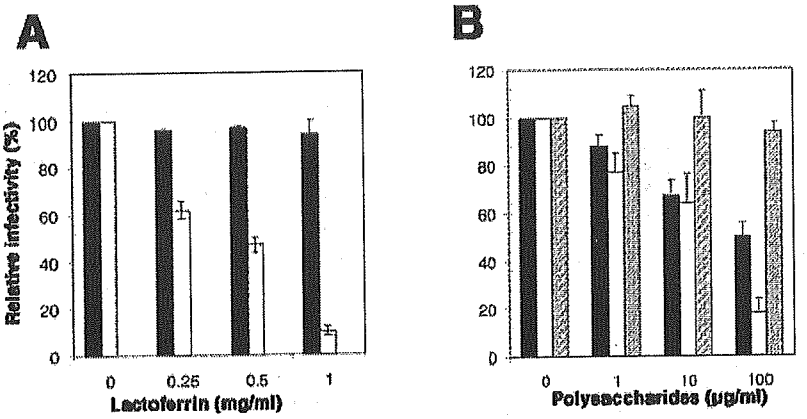

FIG. 4. Effects of bLF and sulfated polysaccharides on the infectivity of VSVΔG*(HBs/M) sample. The VSVΔG*(HBs/M) sample treated with goat anti-VSV serum for 30 min was further treated with bLF (A) or sulfated polysaccharides (B) at 37°C for 30 min and used to infect HepG2 cells. The infected cells were incubated overnight, and the number of GFP-positive cells was counted. Each bar represents the means of relative infectivities (percentages ± standard deviations) calculated from three independent experiments. (A) Relative infectivities of VSVΔG*-G and VSVΔG*(HBs/M) are represented by solid and open bars, respectively. (B) The effects of heparin, dextran sulfate, and dextran are shown by solid, open, and hatched bars, respectively.

cell line, JHH-7, and a human embryonic kidney cell line, 293T, were judged to be clearly susceptible to the VSVΔG*(HBs/M) pseudotype sample. Alexander cells were apparently but less susceptible to the pseudotype sample than HepG2 or JHH-7 cells (data not shown). None of the other cell lines, namely hematopoietic, glioma, or osteosarcoma cell line, tested in this study was apparently, if not at all, susceptible to infection by the pseudotype sample (Table 1). The VSVΔG*-G pseudotype was used as a control to test the susceptibilities of various cell lines to VSV replication. The cell lines used in this study showed different but high degrees of susceptibilities to VSVΔG*-G infection, indicating that a lack of susceptibility to the VSVΔG*(HBs/M) sample was not due to their resistance to VSVΔG*-G.

Effect of chemicals on infection of VSVΔG*(HBs/M) pseudotype samples. Infection of HBV to human hepatocytes was reported to be inhibited by bLF (13). Heparin and other sulfated polysaccharides have also been shown to be strong inhibitors of the binding of HBV to HepG2 cells (49). Thus, we examined effects of bLF, heparin, and dextran sulfate and noticed that infectivity was inhibited by about 90% at 1 mg/ml bLF (Fig. 4A). Sulfated polysaccharides, heparin and dextran sulfate, at 100 μg/ml, also inhibited the infectivity by about 50% and 80%, respectively (Fig. 4B). In contrast, the nonsulfated dextran did not inhibit the infectivity.

We next examined whether a glycosylation of protein(s) on the cell surface is required for HBV infection. For this, HepG2 cells were treated with trypsin just before infection or with tunicamycin 1 day before infection. Treatment with 1.0% trypsin and 10 μg/ml tunicamycin showed about 80% and 70% inhibition of the infectivity, respectively (Fig. 5).

DISCUSSION

In order to prepare VSV pseudotype viruses, the expression of HBs proteins in 293T cells was studied by IFA, FCM, and WB. The S and M proteins are localized mainly in the cytoplasm, and their distribution seemed to be homogeneous inside it, whereas localization of the L protein was granular with its heterogeneous distribution inside the cells (Fig. 1C). This result is consistent with the findings demonstrated by several reporters (32, 47). Localization of S, M, or L protein detected by specific MAbs against HBsAg in hepatocytes of patients with chronic HBV infection reported previously (17) is similar to that observed in 293T cells transfected with corresponding plasmids.

We performed cotransfection experiments using different combinations of the S, M, and L expression plasmids, but the expression levels could not be raised further, as was shown upon individual plasmid transfection (data not shown). This result seems to be different from the result for the recently reported MLV pseudotype. That is, in the report of Sung and Lai (41), the surface expression of L or S protein individually can hardly be detected, whereas about 10% of the surface expression was shown in our experiment when S or M plasmids were transfected independently. We considered that this would be due to a difference between systems employed for the transfection or the use of different expression plasmids to express the HBs proteins. For example, Sung and Lai (41) used retroviral vectors whereas we used expression plasmids containing a β-actin promoter.

After transfection, the transfected cells were infected with VSVΔG*-G for production of VSVΔG*(HBV) pseudotypes that contain the VSV core protein bearing the surface proteins of HBV. As VSV pseudotype titers preliminarily determined had not been sufficiently high (data not shown), the cells detached and suspended in their culture supernatants were sonicated prior to making stocks of the pseudotype samples as described by Tamura et al. (43) to prepare HCV pseudotypes bearing the native form of HCV surface proteins. The infectious titers of the pseudotype samples made with sonication were about five times as high as those of nonsonicated samples (data not shown).

Although the infectivity of the pseudotype was shown to be efficiently neutralized by the anti-HBs S MAbs, the infectivity of the pseudotype sample could not be well neutralized by concentrated human immunoglobulin positive for HBs antibody and available commercially (HBIG) (data not shown). Low titers of HBIG in the neutralization of HBV infection in

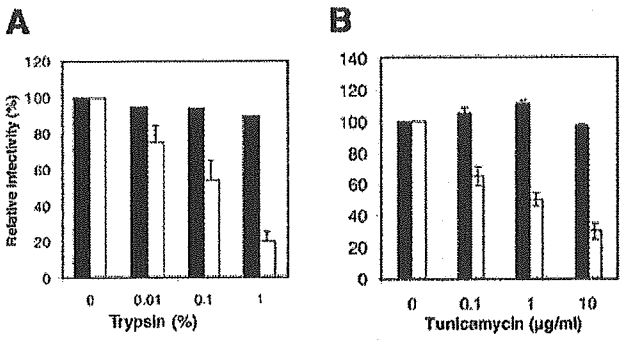

FIG. 5. Effects of trypsin and tunicamycin on the infectivity of the VSVΔG*(HBs/M) pseudotype. HepG2 cells were pretreated with trypsin for 5 min (A) or tunicamycin for 24 h (B) and then inoculated with the VSVΔG*(HBs/M) pseudotype sample. The number of GFP-positive cells was counted after overnight incubation of the cells. Each bar represents the means of relative infectivities (percentages ± standard deviations) calculated from three independent experiments. Relative infectivities of VSVΔG*-G and VSVΔG*(HBs/M) are represented by solid and open bars, respectively.

vitro have already been reported: the HBIG titer is about 2,000 times as low as that of anti-HBs-humanized MAb produced in Ryu et al.'s laboratory (34). Our finding may be consistent with this finding. It is probable that HBIG contains antibodies reacting with the preS1 antigenic determinant, as this region has been considered one of the receptor-binding sites, and that human antibodies may preferentially neutralize native HBV in the blood.

The inhibitory effects of various chemicals, like bLF, tunicamycin, trypsin, and sulfated polysaccharides, on infection of the pseudotype sample was also studied (Fig. 4 and 5). The infectivity of the VSVΔG*(HBs/M) pseudotype was inhibited by bLF in our experiment. The inhibitory effect of bLF, however, seemed to be different from that reported by Hara et al. (13). They reported that preincubation of target cells with bLF is necessary for the inhibition. In contrast, our result is similar to the effect of bLF in HCV infection reported by several groups (13, 43), including Hara's group: preincubation of the cells with bLF before HBs pseudotype infection could not show any inhibitory effect (data not shown). Our results suggest that the bLF-binding site may locate in the preS2 or the S domain of M protein. It is also probable that bLF binds to a region to which neutralizing anti-HBs MAbs also bind. Further study will be necessary to identify a site to which bLF binds.

One of the interesting findings in our study may be that among three HBs proteins, transfection of the expression plasmid DNA for HBs M protein gave the slightly but clearly highest infectious titer of the pseudotype (Fig. 3A) when titrated in HepG2 cells. This finding suggests that M protein of HBV, especially the preS2 region, has an important role in the infection of HBV. This result looks contradictory to some previous reports about its role. For example, Le Seyec et al. (21) reported that most of the preS2 region is dispensable for HBV infection. Cho et al. (5), however, reported the presence of a binding region in the preS2 region for a cellular HBV receptor(s). Namely, the binding region, which contains the N-terminal 5 aa of preS2, potentially contributes to attachment to and entry into target cells (21). Likewise, our results suggest that the preS2 region of M protein may contain a binding region to target cells. The infectious titer of the VSVΔG*(HBs/S) sample was the lowest among all pseudotype samples tested in this study, although all three anti-HBs MAbs neutralized all types of HBs pseudotypes tested.

WB analysis (Fig. 2B) using anti-HBs S MAb showed that WB bands with similar sizes were detected even after transfection of the HBs expression plasmid for the HBs M gene. Thus, it is probable that S protein also is produced in 293T cells transfected with the plasmid for HBs M protein. S protein together with M protein may lead to an efficient production of HBs pseudotype in the HBs/M pseudotype sample. The 21- to 47-aa sequence of preS1 and 120- to 145-aa sequence of preS2 have been reported to be a crucial and an auxiliary cell-binding sites, respectively (28). The presence of the secondary attachment region in HBs S protein has also been reported by Paran et al. (31). However, a direct interaction of natural HBs S protein with hepatocytes or a role of HBs S protein in the penetration of hepatocytes has not been convincingly demonstrated. MAbs used in this study could recognize S-S bonds in the region of HBs S protein, since bands for the specific proteins could be detected upon WB with DTT(−) samples only but not WB with DTT(+) samples, and this region was recognized by the neutralizing MAbs. This finding suggests that a receptor-binding site as well as a MAb-binding site in HBV surface proteins may be created by the S-S bond. In other words, the S-S bond in the S protein or S domain in M and L proteins may play an important role in entry of HBV into target cells.

Although there seem to be some contradictory results in our study to those reported previously, the discrepancies might be attributable to the difference in systems to detect HBV infection, such as whether inoculating samples are native virus derived from the blood or VSV pseudotypes, whether target cells are primary hepatocytes or established cell lines, or whether titers of inoculating viruses are high or low. Properties of HBs proteins produced in hepatocytes in vivo or those produced in 293T cells in vitro may be different. If titers of inoculating viruses are detected to be different, a region of HBs gene necessary for infection or a susceptibility of cells to HBV can be determined differently.

In conclusion, we considered that VSV pseudotypes bearing the surface proteins of HBV could be prepared. These pseudotypes most efficiently infected the human hepatoma cell lines HepG2 and JHH-7 among establish human cell lines tested. To our knowledge, this is the first report of production of VSV pseudotype virus bearing HBs proteins. The VSVΔG*(HBV) pseudotypes might be useful in a safe and rapid assay system to clarify early steps of HBV infection. This system may also contribute to screening and development of antiviral drugs that interfere with HBV infection.

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The Synthetic Peptide Derived from the NH_2-terminal Extracellular Region of an Orphan G Protein-coupled Receptor, GPR1, Preferentially Inhibits Infection of X4 HIV-1*

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Several G protein-coupled receptors (GPCRs) serve as co-receptors for entry of human immunodeficiency virus type 1 (HIV-1) into target cells. Here we report that a synthetic peptide derived from the NH_2-terminal extracellular region of an orphan GPCR, GPR1 (GPR1ntP-(1–27); MEDLEETLFEEFENYSYDLDYYSLESC), inhibited infection of not only an HIV-1 variant that uses GPR1 as a co-receptor, but also X4, R5, and R5X4 viruses. Among these HIV-1 strains tested, viruses that can utilize CXCR4 as their co-receptors were preferentially inhibited. Inhibition of early steps in X4 virus replication was also detected in the primary human peripheral blood lymphocytes. GPR1ntP-(1–27) directly interacted with recombinant X4 envelope glycoprotein (rgp120). This interaction was neither inhibited nor enhanced by the soluble CD4 (sCD4) but inhibited by the anti-third variable (V3) loop-specific monoclonal antibody and heparin known to bind to the V3 loop. Although the conformational changes in gp120, including the V3 loop, have been reported to be required for its interaction with a co-receptor after binding of gp120 to CD4, it has also been reported that the V3 loop is already exposed on the surface of virions before interaction with CD4. We found that GPR1ntP-(1–27) blocked binding of virus to the cells, and this peptide equally bound to rgp120 in the presence or absence of sCD4. Because we detected the binding of GPR1ntP-(1–27) to the highly purified virions even in the absence of sCD4, GPR1ntP-(1–27) probably recognized the V3 loop exposed on the virions, and this interaction was responsible for the anti-HIV-1 activity of GPR1ntP-(1–27).

HIV-1[1] is the causative agent of AIDS (1, 2). CD4 glycoprotein as a receptor and GPCRs as co-receptors are required for the entry of HIV-1, and the cell tropism of HIV-1 is mainly determined by its ability to use co-receptors expressed on the target cells (3). The major co-receptor for R5 viruses is CCR5, of which ligands are CC chemokines RANTES (regulated on activation normal T-cell expressed and secreted), MIP1α, and MIP1β. On the other hand, the major co-receptor for X4 viruses is CXCR4, of which ligand is the CXC chemokine SDF-1. Dual-tropic viruses (R5X4 viruses) can efficiently utilize both of these co-receptors (4–9).

In addition, primary human brain-derived fibroblast-like cells (BT-cells) are isolated from microvessel segments derived from autopsy of human brain tissue, are positive for CD4 and highly susceptible to HIV-1 variants, but are resistant to R5, X4, and R5X4 HIV-1 strains (10, 11). BT-cells are thought to be originated from smooth muscle cells or pericytes. We have previously shown that the substitution of Pro to Ser at the Gly-Pro-Gly-Arg sequence in the V3 loop of envelope glycoprotein (gp120) is responsible for the BT-cell tropism of the HIV-1 variants (10, 12). We have recently identified orphan GPCRs, GPR1 and RDC1, as co-receptors for the HIV-1 variants *in vitro* (13, 14). Not only HIV-1 variants, but also several strains of HIV-2 and simian immunodeficiency virus have been shown to utilize GPR1 and RDC1 as their co-receptors (13–15).

During the course of our previous experiments to generate anti-GPR1 antibody (α-GPR1) by immunizing rabbits with a synthetic peptide derived from the NH_2-terminal extracellular region (NH_2-ECR) of GPR1 (GPR1ntP-(1–27)), we found unexpectedly that not only α-GPR1 but also GPR1ntP-(1–27) was able to block GPR1-mediated infection of BT-cells with HIV-1 variants. Because it has been demonstrated that the NH_2-ECRs of the co-receptors play an important role in the interaction with gp120 and a subsequent infection steps (16–21), we speculated that GPR1ntP-(1–27) might inhibit interaction of gp120 (HIV-1 variants) with its co-receptor, GPR1, on target cell membrane.

In the present study, we tested anti-HIV-1 activity of GPR1ntP-(1–27) by several assays, including focal infectivity assay, HIV-1 p24 Gag detection assay, PCR assay, and syncytium formation assay. These assays all demonstrated an inhibitory activity of GPR1ntP-(1–27) against HIV-1 infection. Surprisingly, anti-HIV-1 activity of GPR1ntP-(1–27) was detected not only in HIV-1 variants that utilize GPR1 as a co-receptor, but also in genetically diverse isolates, including X4, R5, and

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; GPCR, G protein-coupled receptor; BT-cell, primary human brain-derived fibroblast-like cell; NH_2-ECR, NH_2-terminal extracellular region; FCS, fetal calf serum; PBL, peripheral blood lymphocyte; mAb, monoclonal antibody; sCD4, soluble CD4; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; bd, binding domain.

TABLE I
Amino acid sequences of the synthetic peptides derived from the NH_2-terminal extracellular region of GPCRs

GPCR	Peptide	Length	Amino acid sequence	Molecular weight	Net charge
GPR1	GPR1ntP-(1–27)	27	MEDLEETLFEEFENYSYDLDYYSLESC[a]	3378	−10
CXCR4	X4ntP-(1–28)	28	MEGISIYTSDNYTEEMGSGDYDSMKEPC	3007	−6
CCR3	R3ntP-(1–24)	24	MTTSLDTVETFGTTSYYDDVGLLC	2766	−4
CCR5	R5ntP-(1–20)	20	MDYQVSSPIYDINYYTSEPC	2389	−3
GPR1	GPR1ntP-(1–13)	13	MEDLEETLFEEFE	1661	−7
GPR1	GPR1ntP-(8–20)	13	LFEEFENYSYDLD	1684	−3
GPR1	GPR1ntP-(15–27)	13	YSYDLDYYSLESC	1621	−3
GPR1	GPR1ntP-(Y/A)	27	MEDLEETLFEEFENASADLDAASLESC[b]	3010	−10

[a] As described in the Introduction, the COOH-terminal cysteine residue of GPR1ntP-(1–27) is introduced into the sequence for the purpose of conjugation of the keyhole limpet hemocyanine to use as an immunogen for antibody production.
[b] All tyrosine residues in GPR1ntP-(1–27) were substituted to alanine (underlined) in GPR1ntP-(Y/A).

R5X4 viruses. Our findings suggest that synthetic peptides derived from the NH_2-ECR of GPR1 are novel candidates for the development of GPCR-based and peptide-based agents to inhibit HIV-1 infection.

EXPERIMENTAL PROCEDURES

Cells—NP-2 cells were derived from a human glioma and kindly provided by Dr. T. Kumanishi (Niigata University, Niigata, Japan). NP-2/CD4 and NP-2/CD4 cells stably expressing human GPCRs (NP-2/CD4/GPCRs, such as NP-2/CD4/CCR3, NP-2/CD4/CCR5, NP-2/CD4/CXCR4, and NP-2/CD4/GPR1) as indicator cells for HIV-1 infection were described previously (13–15, 22, 23). NP-2/CD4 and NP-2/CD4/GPCRs cells were maintained in Eagle's minimum essential medium containing 10% fetal calf serum (FCS). The human T-cell lines C8166 (24), MOLT-4 clone 8 (25), and MOLT4/IIIB (26) were maintained in RPMI 1640 medium supplemented with 10% FCS (RPMI/FCS). Peripheral blood lymphocytes (PBLs) were isolated from the blood of healthy subjects by Ficoll-Paque gradient centrifugation. PBLs were stimulated with phytohemagglutinin prior to HIV-1 infection and cultured in RPMI/FCS and 100 units/ml of recombinant interleukin-2 (Roche Applied Science). All cells were maintained at 37 °C in a humidified, 5% CO_2 atmosphere.

HIV-1 Strains—The GUN-1WT strain is a clinical isolate and can infect both T-cell lines and macrophages, but not BT-cells (10, 12, 27). In contrast, the variant of GUN-1WT strain can infect the BT-cells and T-cell lines (10, 12). Similar to the variant of GUN-1WT strain, GUN-1Ser strain, which had been prepared by the ligation and transfection of cloned HIV-1 (GUN-1WT) DNA fragments containing a mutation of proline to serine made by the site-directed mutagenesis at the GPGR sequence in the V3 loop, can infect BT-cells as well as T-cell lines (10, 12). The GUN-1WT, GUN-1Ser, and IIIB (28) strains of HIV-1 were produced by persistently infected MOLT-4 cells. The BaL (29) and SF162 (30) strains of HIV-1 were produced by infected PBLs. The primary HIV-1 strains, GUN-4 and GUN-14, were isolated from Japanese hemophilia patients as described previously (12). To prepare virus samples, culture supernatants containing HIV-1 were harvested, centrifuged at a low speed, and passed through 0.45-μm pore size cellulose acetate syringe filters (Gellman Science, Ann Arbor, MI). The virus samples were stored at −80 °C until use.

Synthetic Peptides—All synthetic peptides used in this study were purchased from Sawady Technology (Tokyo, Japan). The purity of the peptides was more than 95%. The amino acid sequences of the synthetic peptides used in this study are shown in Table I.

Antibodies—Sera from patients infected with HIV-1 were heat-inactivated (56 °C, 30 min) before use. The polyclonal rabbit antibodies to GPR1ntP-(1–27) (α-GPR1), and X4ntP-(1–28) (α-CXCR4) were generated and purified through peptide-coupled affinity columns. Mouse ascites containing the monoclonal antibody (mAb) 0.5β, which recognizes an epitope in the V3 domain and specifically neutralizes infection of HIV-1 IIIB strain (31), was kindly provided by Dr. S. Matsushita (Kumamoto University, Kumamoto, Japan). The mouse ascites containing mAb against the HIV-1 p24 Gag ($p24^{Gag}$) protein were obtained from Immunoprobe Co. (Saitama, Japan). The sheep antibody against soluble CD4 (sCD4) was obtained from the AIDS Reagent Project of the United Kingdom Medical Research Council (Potters Bar, United Kingdom). The mAb to human CD4 (NU-TH/I), which inhibits HIV-1 gp120 binding to human CD4 and recognizes a discontinuous conformational epitope corresponding to the complementary-determining region 2 and 3 (CDR2 and CDR3) of CD4 (32), was purchased from Nichirei, Tokyo, Japan.

HIV-1 Infectivity Assay—To determine HIV-1 infectivity, a focal infectivity assay was performed as described previously (33–37). Namely, NP-2/CD4 cells expressing one of GPCRs were seeded at 2×10^4 per well into 24-well tissue culture plates (Corning, Acton, MA), infected with 200 μl of the virus (1,000–2,500 focus-forming units per ml) in the presence or absence of one of the synthetic peptides. After 2 h at 37 °C, the cells were washed, fresh culture medium was added, and the cultures were incubated for 48 h at 37 °C. The cells were fixed with methanol for 10 min at room temperature, rinsed with PBS, and then incubated with anti-HIV-1 sera from AIDS patients diluted 1:300 with 3% bovine serum albumin (w/v) in PBS (blocking buffer A) for 2 h at room temperature. After being washed with PBS, cells were incubated with horseradish peroxidase-labeled antibody to human IgG (Dako, Glostrup, Denmark) diluted 1:200 with blocking buffer A for 1 h at room temperature, washed with PBS, and rinsed with Tris-buffered saline. To develop the color, cells were reacted in the dark with a solution of aminoethyl carbazol (Sigma) and H_2O_2 in acetate buffer (pH 5.0) for 20 min at room temperature, rinsed with water, air dried, and examined under a microscope. To determine the inhibitory activity of peptide to HIV-1 infection (inhibition (%)), the following formula was used: [(the number of foci with no peptide − the number of foci with peptide)/the number of foci with no peptide] × 100%.

To examine the effect of GPR1ntP-(1–27) on the infectivity of HIV-1 primary isolates, the level of $p24^{Gag}$ protein in culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (38). Azido-3′-deoxythymidine was purchased from Sigma.

PCR Assay to Detect Reverse-transcribed HIV-1 DNA—The virus preparation (IIIB strain) was treated with RNase-free DNase I (10 units/ml, Roche Applied Science) for 30 min at 37 °C to remove contaminating viral DNA or cellular DNA. DNase I-treated IIIB virus was incubated with one of the synthetic peptides for 1 h at 37 °C, and inoculated onto MOLT-4 cells (5×10^6) or PBLs (5×10^5) for 2 h at 37 °C. The cells were washed, and fresh medium was added. After incubation for 20 h, the cells were washed and lysed with 10 mM Tris-HCl (pH 8.3) containing 1 mM EDTA, 0.45% Nonidet P-40 (Sigma), 0.45% Tween 20 (Sigma), and 0.2 mg/ml proteinase K (Sigma). The cell lysates were incubated for 2 h at 52 °C, heated for 10 min at 96 °C to inactivate proteinase K, and used as templates for the subsequent PCR analysis to detect the formation of reverse-transcribed HIV-1 DNA within the cells. PCR was performed with HIV-1 *gag*-specific primers, SK38 and SK39 (39) (nucleotide sequence: SK38, 5′-AAGGGGAAGTGACATAGCAG-3′; SK39, 3′-GGACCAACAAGGTTTCTGTC-5′) in a PerkinElmer Life Sciences Cycler under the following conditions: 1 cycle at 95 °C for 9 min, 30 cycles at 94 °C for 1 min, 60 °C for 45 s, 72 °C for 1 min, and one cycle at 72 °C for 5 min. The human β-globin gene primers, KM29 and KM38 (nucleotide sequence: KM29, 5′-GGTTGGCCAATCTACTCCCAGG-3′; KM38, 5′-TGGTCTCCTTAAACCTGTCTTG-3′) (40) were used as an internal control. DNA amplification was performed in the following conditions: 1 cycle at 95 °C for 9 min, 30 cycles at 94 °C for 1 min, 60 °C for 45 s, 72 °C for 1 min, and one cycle at 72 °C for 5 min. The PCR products were visualized by electrophoresis through 2% agarose gels containing 0.5 μg/ml ethidium bromide.

HIV-1 Cell Binding Assay—HIV-1-cell binding assay was essentially done according to the protocol described by Valenzuela *et al.* (41). MOLT-4 cells (6×10^6) were incubated with HIV-1 (IIIB strain) (200 ng of $p24^{Gag}$) for 1 h at 37 °C in RPMI/FCS (final volume, 0.5 ml). When indicated, viral preparation was incubated with GPR1ntP-(1–27), GPR1ntP-(Y/A), 0.5β, or heparin, while the cells were incubated with anti-CD4 mAb, NU-TH/I, before binding. After incubation of the cells