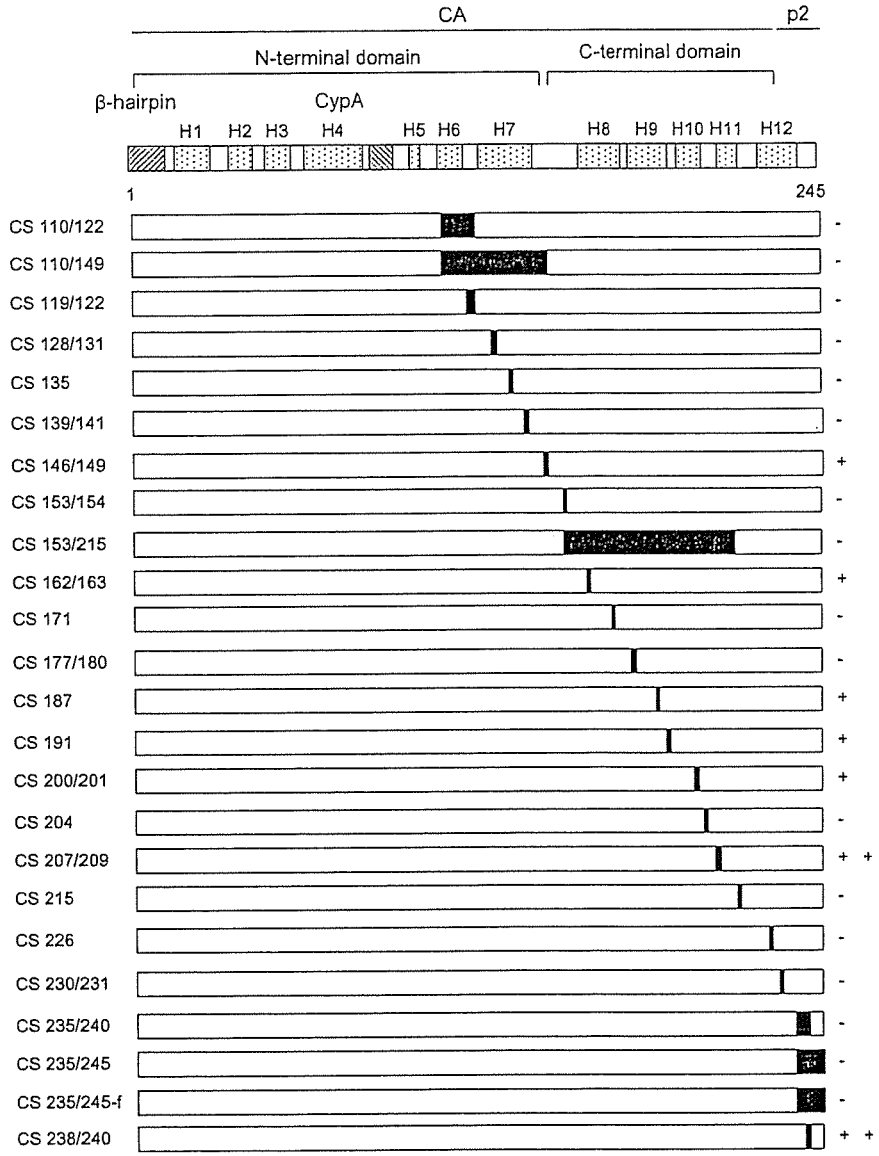

Fig. 1 (*continued*).

SIVmac on the virus [20]. However, the replication-competent virus, designated NL-CAi2, did not grow at all in monkey HSC-F cells [20]. These results prompted us to construct and characterize *gag* (CA-p2)-hybrid viruses more extensively. Structural analyses have already revealed the unique features of HIV-1 CA and provided a model for the intact protein [29,30]. Based on these findings, we introduced SIVmac *gag* sequences into all functionally important domains of HIV-1 CA-p2 as small or large insertions. As shown in Fig. 1, 49 recombinants in total were finally constructed.

Upon transfection into 293T cells, all the recombinants (Fig. 1) produced progeny virions at a level comparable to the wt clone (40–120%), as judged by RT production. These results indicated that the recombinants may have no major late replication defects in cells. We then asked whether these recombinants display multi-cycle infectivity in human and simian cells. All the viruses (5×10^6 RT units of each) prepared from transfected 293T cells were inoculated into human lymphocytic M8166 cells (1×10^6 cells), and their growth kinetics were determined. Representative growth properties in the cells of the recombinants are shown in Fig. 2, and all the data obtained from the infectivity assay are summarized in Fig. 1. Out of the 49 recombinants constructed, 18 (CS2/15, CS9/15, CS13/15, CS47, CS47/52, CS47/54, CS58/61, CS86/93, CS86/100, CS86/122, CS110/112-119/122, CS146/149, CS162/163, CS187, CS191, CS200/201, CS207/209 and CS238/240) were found to be infectious toward M8166 cells. Six of the 18 clones (CS13/15, CS47, CS86/93, CS86/100, CS207/209 and CS238/240) grew similarly well to the wt virus. These results showed that SIVmac *gag* sequences can be inserted into various parts of the corresponding HIV-1 CA-p2 region without abolishing the infectivity of the virus. However,

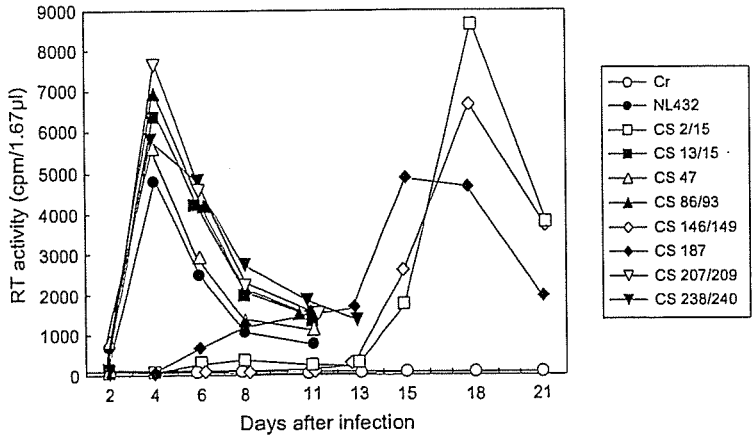

Fig. 2. Growth kinetics in M8166 cells of chimeric viruses. Cells were infected with cell-free viruses as described in the text, and virus replication was monitored at intervals by RT production in the culture supernatants. Input viruses were prepared from 293T cells transfected with 20 μg of the clones indicated on the right. Cr, pUC19.

almost all recombinants carrying an insertion in the α-helix grew poorly or not at all (Fig. 1). We then inoculated all the recombinants (1×10^7 RT units for each) into monkey lymphocytic HSC-F cells (1×10^7 cells) using SIVmac prepared from 293T cells transfected with pMA239 [2] as a positive control. No recombinants were found to be infectious for HSC-F cells (data not shown).

3.2. Biochemical characterization of gag-chimeric clones

Fourteen recombinants were selected and examined for their biochemical properties in cells. These included non-infectious (CS5/15, CS39/47, CS86/112-119/122, CS153/154, CS204 and CS235/245-f), poorly infectious (CS9/15 and CS146/149) and highly infectious (CS13/15, CS47, CS86/93, CS86/100, CS207/209 and CS238/240) clones for M8166 cells. The insertion sites of SIVmac *gag* sequences in these recombinants are located throughout the CA-p2 region of HIV-1 (Fig. 1).

First, the Gag expression in cells and the Gag profile in virions were confirmed. Because RT production in 293T cells transfected with the recombinants was fairly normal, no major defects were expected to be observed. 293T cells were transfected with various clones, and on day 2 post-transfection, cells were harvested for Western blot analysis. As shown in Fig. 3, no clear abnormality was seen for the recombinants tested, except CS235/245-f. Consistent with the insertion

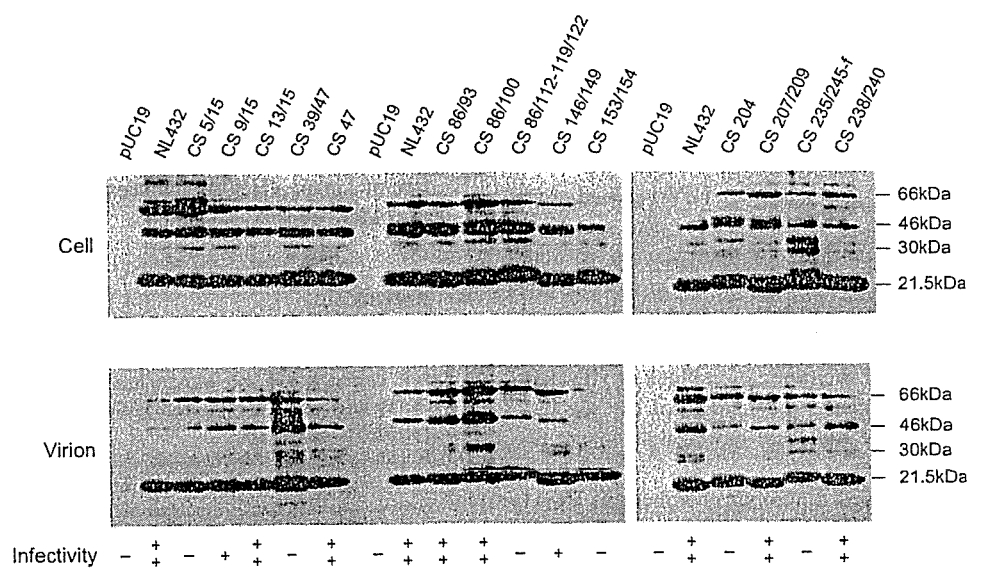

Fig. 3. Western blot analysis of chimeric viruses. Cell and virion lysates were prepared from 293T cells transfected with various clones (20 μg) indicated at the top, as described previously [20,25,26]. Each sample was then subjected to Western blot analysis using a human anti-HIV-1 antiserum as reported before [19]. Results obtained from three independent experiments are shown. The infectivity of viruses for M8166 cells is given as ++ (wt growth), + (retarded growth) and − (no growth) at the bottom. Protein size is shown on the right. Cell, lysates from transfected cells; virion, virion lysates.

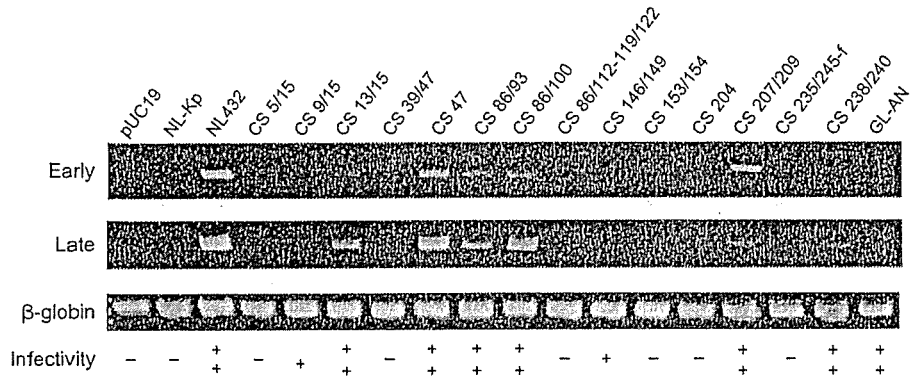

Fig. 4. PCR analysis of chimeric viruses. M8166 cells were infected with cell-free viruses, as described in the text. Input viruses were prepared from 293T cells transfected with 20 μg of the clones indicated at the top. DNA was extracted from infected cells on day 2 post-infection, and subjected to PCR analysis using the early and late primers as described previously [27]. To ascertain an approximate equality of DNA amount in each sample, β-globin gene was amplified by PCR [16,28]. The infectivity of viruses for M8166 cells is given as ++ (wt growth) + ,(retarded growth) and − (no growth) at the bottom.

that CS235/245-f carries (Fig. 1), it produced a considerable amount of Gag processing intermediates in cells, 33- and 34-kDa proteins, reported by us [31]. However, in the virions of CS235/245-f, the insertional effect was relatively small.

Second, the viral DNA synthesis in cells was monitored. M8166 cells were infected with the recombinants as above using an Env-minus HIV-1 mutant (NL-Kp) [17] and wt HIV-2 (GL-AN) [16] as negative controls. On day 2 post-infection, cells were harvested for PCR analysis. As shown in Fig. 4, parallel with the high infectivity in M8166 cells, the recombinants directed the synthesis of viral DNA. Viral specific DNA was readily detected only in cells infected with wt HIV-1 (NL432) or highly infectious recombinant viruses. We then monitored the synthesis of viral DNA in HSC-F cells infected with the same viruses as above. However, definite data, as evaluated by the PCR method here, were difficult to obtain probably due to the relatively low susceptibility of the cells to viruses.

4. Discussion

In this study, we have generated 18 HIV-1 based *gag*-chimeric viruses that are capable of productive and spreading infection in M8166 cells. These recombinant viruses, particularly the highly infectious ones, would be useful to construct an HIV-1 that is tropic for monkey cells to establish an animal infection model in the near future. They are also important as tools to analyze the basis for the replication block of HIV-1 in monkey cells. Understanding the mechanism of the species-specific tropism of HIV/SIV may add new insight to the research field of basic virology.

Although we have obtained a number of infectious HIV-1 carrying SIVmac *gag* sequences, none of them were able to grow in HSC-F cells, as monitored by virion-associated RT production. The defective site(s) in the cells of our recombinants is currently unclear. However, on the basis of the data presented here, it is quite possible that they have an early replication defect at the postentry step. Also, we have previously shown that the block for HIV-1 replication in monkey cells resides in the process of uncoating and/or reverse transcription [5]. Furthermore, the viral proteins responsible for the block appear to be Gag and an undetermined viral protein(s) [5–7]. Taken altogether, we may conclude that there is a viral factor, other than Gag CA, critical for the escape from the replication restriction and for viral DNA synthesis in monkey cells. We are now constructing a new series of chimeric viral clones to substantiate this hypothesis.

Acknowledgements

We thank Ms Kazuko Yoshida for editorial assistance. This work was supported by the following grants. (1) From the Japan Society for the Promotion of Science: a Research Fellowship for Young Scientists (17-0561), a Grant-in-Aid for Exploratory Research (14657076) and a Grant-in-Aid for Scientific Research (B)(14370103). (2) From the Ministry of Education, Culture, Sports, Science and Technology of Japan: a Grant-in-Aid for Scientific Research on Priority Areas (2) (16017270). (3) From the Ministry of Health, Labour and Welfare of Japan: a Health Sciences Research Grant (Research on HIV/AIDS 16150301).

References

[1] R.C. Desrosiers, Nonhuman lentiviruses, in: D.M. Knipe, P.M. Howley, D.E. Griffin, R.A. Lamb, M.A. Martin, B. Roizman, S.E. Straus (Eds.), Fields Virology, Lippincott Williams and Wilkins, Philadelphia, 2001, pp. 2095–2121.

[2] R. Shibata, M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, A. Adachi, Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells, J. Virol. 65 (1991) 3514–3520.

[3] S. Sakuragi, R. Shibata, R. Mukai, T. Komatsu, M. Fukasawa, H. Sakai, J. Sakuragi, M. Kawamura, K. Ibuki, M. Hayami, A. Adachi, Infection of macaque monkeys with a chimeric human and simian immunodeficiency virus, J. Gen. Virol. 73 (1992) 2983–2987.

[4] R. Shibata, A. Adachi, SIV/HIV recombinants and their use in studying biological properties, AIDS Res. Hum. Retrov. 8 (1992) 403–409.

[5] R. Shibata, H. Sakai, M. Kawamura, K. Tokunaga, A. Adachi, Early replication block of human immunodeficiency virus type 1 in monkey cells, J. Gen. Virol. 76 (1995) 2723–2730.
[6] K. Uberla, C. Stahl-Henning, D. Bottiger, K. Matz-Rensing, F.J. Kaup, J. Li, W.A. Haseltine, B. Fleckenstein, G. Hunsmann, B. Oberg, Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 8210–8214.
[7] T. Dorfman, H.G. Gottlinger, The human immunodeficiency virus type 1 capsid p2 domain confers sensitivity to the cyclophilin-binding drug SDZ NIM811, J. Virol. 70 (1996) 5751–5757.
[8] J. Sakuragi, H. Sakai, M. Kawamura, K. Tokunaga, S. Ueda, A. Adachi, Generation and characterization of a host cell-dependent *gag* gene mutant of human immunodeficiency virus type 1, Virology 212 (1995) 251–254.
[9] R.A. Furuta, R. Shimano, T. Ogasawara, R. Inubushi, K. Amano, H. Akari, M. Hatanaka, M. Kawamura, A. Adachi, HIV-1 capsid mutants inhibit the replication of wild-type virus at both early and late infection phase, FEBS Lett. 415 (1997) 231–234.
[10] M. Kawamura, R. Shimano, R. Inubushi, H. Akari, A. Adachi, Early function of HIV-1 Gag proteins is cell-dependent, Biochem. Bioph. Res. Co. 248 (1998) 899–903.
[11] K.-B. Koh, M. Miyaura, A. Yoshida, A. Sakurai, M. Fujita, A. Adachi, Cell-dependent *gag* mutants of HIV-1 are crucially defective at the stage of uncoating/reverse transcription in non-permissive cells, Microbes Infect. 2 (2000) 1419–1423.
[12] C.M. Owens, B. Song, M.J. Perron, P.C. Yang, M. Stremlau, J. Sodroski, Binding and susceptibility to postentry restriction factors in monkey cells are specified by distinct regions of the human immunodeficiency virus type 1 capsid, J. Virol. 78 (2004) 5423–5437.
[13] T. Hatziioannou, S. Cowan, U.K. von Schwedler, W.I. Sundquist, P.D. Bieniasz, Species-specific tropism determinants in the human immunodeficiency virus type 1 capsid, J. Virol. 78 (2004) 6005–6012.
[14] Y. Ikeda, L.M.J. Ylinen, M. Kahar-Bador, G.J. Towers, Influence of *gag* on human immunodeficiency virus type 1 species-specific tropism, J. Virol. 78 (2004) 11816–11822.
[15] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.
[16] F. Ueno, H. Shiota, M. Miyaura, A. Yoshida, A. Sakurai, J. Tatsuki, A.H. Koyama, H. Akari, A. Adachi, M. Fujita, Vpx and Vpr proteins of HIV-2 up-regulate the viral infectivity by a distinct mechanism in lymphocytic cells, Microbes Infect. 5 (2003) 387–395.
[17] A. Adachi, N. Ono, H. Sakai, K. Ogawa, R. Shibata, T. Kiyomasu, H. Masuike, S. Ueda, Generation and characterization of the human immunodeficiency virus type 1 mutants, Arch. Virol. 117 (1991) 45–58.
[18] M. Fujita, A. Sakurai, A. Yoshida, M. Miyaura, A.H. Koyama, K. Sakai, A. Adachi, Amino acid residues 88 and 89 in the central hydrophilic region of human immunodeficiency virus type 1 Vif are critical for viral infectivity by enhancing the steady-state expression of Vif, J. Virol. 77 (2003) 1626–1632.
[19] M. Fujita, H. Akari, A. Sakurai, A. Yoshida, T. Chiba, K. Tanaka, K. Strebel, A. Adachi, Expression of HIV-1 accessory protein Vif is controlled uniquely to be low and optimal by proteasome degradation, Microbes Infect. 6 (2004) 791–798.
[20] M. Fujita, A. Yoshida, M. Miyaura, A. Sakurai, H. Akari, A.H. Koyama, A. Adachi, Cyclophilin A-independent replication of a human immunodeficiency virus type 1 isolate carrying a small portion of the simian immunodeficiency virus SIV_{MAC} *gag* capsid region, J. Virol. 75 (2001) 10527–10531.
[21] J.S. Lebkowski, S. Clancy, M.P. Calos, Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression, Nature 3 (1985) 169–171.
[22] H. Akari, T. Fukumori, S. Iida, A. Adachi, Induction of apoptosis in Herpesvirus saimiri-immortalized T lymphocytes by blocking interaction of CD28 with CD80/CD86, Biochem. Bioph. Res. Co. 263 (1999) 352–356.
[23] T. Folks, S. Benn, A. Rabson, T. Theodore, M.D. Hoggan, M. Martin, M. Lightfoote, K. Sell, Characterization of a continuous T-cell line susceptible to the cytopathic effects of acquired immune deficiency syndrome (AIDS)-associated retrovirus, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 4539–4543.
[24] R.L. Willey, D.H. Smith, L.A. Lasky, T.S. Theodore, P.L. Earl, B. Moss, D.J. Capon, M.A. Martin, In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity, J. Virol. 62 (1988) 139–147.
[25] H. Akari, S. Arold, T. Fukumori, T. Okazaki, K. Strebel, A. Adachi, Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation, J. Virol. 74 (2000) 2907–2912.
[26] H. Akari, T. Fukumori, A. Adachi, Cell-dependent requirement of human immunodeficiency virus type 1 gp41 cytoplasmic tail for Env incorporation into virions, J. Virol. 74 (2000) 4891–4893.
[27] K.-B. Koh, M. Fujita, A. Adachi, Elimination of HIV-1 plasmid DNA from virus samples obtained from transfection by calcium-phosphate co-precipitation, J. Virol. Methods 90 (2000) 99–102.
[28] H.M. Bauer, Y. Ting, C.S. Grier, J.C. Chambers, C.J. Tashiro, J. Chimera, A. Reingold, M.M. Manos, Genital human papilloma virus infection in female university students as determined by a PCR-based method, J. Am. Med. Assoc. 265 (1991) 472–477.
[29] T.R. Gamble, F.F. Vajdos, S. Yoo, D.K. Worthylake, M. Houseweart, W.I. Sundquist, C.P. Hill, Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid, Cell 87 (1996) 1285–1294.
[30] T.R. Gamble, S. Yoo, F.F. Vajdos, U.K. von Schwedler, D.K. Worthylake, H. Wang, J.P. McCutcheon, W.I. Sundquist, C.P. Hill, Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein, Science 278 (1997) 849–853.
[31] H. Akari, M. Fujita, S. Kao, M.A. Khan, M. Shehu-Xhilaga, A. Adachi, K. Strebel, High level expression of human immunodeficiency virus type-1 Vif inhibits viral infectivity by modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site, J. Biol. Chem. 279 (2004) 12355–12362.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Microbes and Infection 8 (2006) 10–15
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Comparative study on the structure and cytopathogenic activity of HIV Vpr/Vpx proteins

Boonruang Khamsri [a], Fumiko Murao [b], Akiko Yoshida [a], Akiko Sakurai [a], Tsuneo Uchiyama [a], Hiroki Shirai [c,1], Yo Matsuo [c], Mikako Fujita [a,*], Akio Adachi [a]

[a] *Department of Virology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan*
[b] *Department of Ophthalmology and Visual Neuroscience, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan*
[c] *Computational Proteomics Team, Protein Research Group, RIKEN Genomic Sciences Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan*

Received 24 April 2005; accepted 19 May 2005

Available online 08 August 2005

Abstract

The three-dimensional (3-D) structure of human immunodeficiency virus type 2 (HIV-2) Vpr/Vpx was predicted by homology modeling based on the NMR structure of human immunodeficiency virus type 1 (HIV-1) Vpr. The three proteins similarly have three major amphipathic α-helices. In contrast to HIV-1 Vpr, Vpr/Vpx of HIV-2 have a long N-terminal loop and clustered prolines in the second half of the C-terminal loop. HIV-2 Vpx uniquely contains a long region between the second and third major helices, and bears several glycines in the first half of the C-terminal loop. Instead of the glycines, there is a group of hydrophilic amino acids and arginines in the corresponding regions of the two Vprs. To compare the cytopathogenic potentials of HIV-1 Vpr and HIV-2 Vpr/Vpx, we examined the production of luciferase as a marker of cell damage. We further analyzed the characteristics of cells transduced with *vpr/vpx* genes driven by an inducible promoter. The results obtained clearly show that structurally similar, but distinct, HIV Vpr/Vpx proteins are detrimental to target cells.

Keywords: HIV-1; HIV-2; Vpr; Vpx; Homology modeling; Cytopathogenic activity

1. Introduction

All human and simian immunodeficiency viruses (HIVs and SIVs) isolated so far carry an accessory gene, *vpr*, in their genomes [1]. HIV type 2 (HIV-2) and SIVs isolated from rhesus (SIVmac) and sooty mangabey (SIVsm) monkeys constitute an independent sub-group (HIV-2 group) within primate immunodeficiency virus groups, and carry a *vpx* gene in addition to *vpr* [2]. Recently, SIVs from the mandrill (SIVmnd-2), red-capped mangabey monkey (SIVrcm) and drill (SIVdrl) have been reported to have both *vpr* and *vpx*, like viruses of the HIV-2 group [3–5]. A complete set of SIVmac *vpr* and *vpx* is required to cause AIDS efficiently in rhesus monkeys [6–8]. The *vpr* and *vpx* share considerable sequence similarity [2,5], and encode small proteins of approximately 100 amino acids. The three-dimensional (3-D) structure of HIV-1 Vpr determined by NMR is characterized by three major α-helices surrounded by N and C-terminal loops [9]. Structural analyses of Vpr/Vpx proteins other than HIV-1 Vpr, however, have not yet been carried out.

HIV-1 Vpr has been demonstrated to display cytopathogenic activities such as cell cycle arrest at the G_2 phase (G_2 arrest) [10–15] and apoptosis [15–17]. It was reported that the Vpr of HIV-2 group arrests cells at the G_2 phase [14,18–21], while Vpx does not [13,14,18,19,21]. The role of Vpr/Vpx of the HIV-2 group for apoptosis has not yet been well documented. While the virological significance of G_2 arrest and apoptosis induced by Vpr remains unclear, the ability to induce G_2 arrest is conserved among various primate immunodeficiency viruses [19,20,22]. The cytopathogenic potential of

* Corresponding author. Tel.: +81 88 633 9232; fax: +81 88 633 7080.
E-mail address: mfujita@basic.med.tokushima-u.ac.jp (M. Fujita).
[1] Present address: Molecular Medicine Laboratories, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan.

1286-4579/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.micinf.2005.05.020

Vpr/Vpx, however, has not been compared yet under the same experimental conditions.

In this study, the 3-D structure and cytopathogenic activity of HIV-1 Vpr and HIV-2 Vpr/Vpx were compared. To analyze the framework of HIV-2 Vpr/Vpx, homology modeling based on the HIV-1 Vpr structure was performed. To evaluate their cytopathogenic activity, cells transiently or stably transfected with various expression vectors for HIV Vpr/Vpx were characterized biochemically and biologically. We demonstrate here that HIV-1 Vpr and HIV-2 Vpr/Vpx are structurally quite similar, but have distinct characteristics, and also that these proteins are detrimental to target cells.

2. Materials and methods

2.1. Homology modeling

Sequence alignment of HIV-1 Vpr and HIV-2 Vpr/Vpx was performed by the Clustal W program [23]. On the basis of this alignment, the 3-D structure of HIV-2 Vpr/Vpx was predicted from the NMR structure of HIV-1 Vpr (Protein Data Bank (PDB) code 1ESX) [9] by the MODELLER 6v2 program [24], and a diagram was generated by RASMOL software [25]. The amino acid sequence of HIV-1 Vpr and those of HIV-2 Vpr/Vpx are from HIV-1 P896 (GenBank accession no. U39362) [26] and HIV-2 GH-1 (GenBank accession no. M30895) [27] isolates, respectively. The sequences of *vpr/vpx* in HIV-2 GH-1 are identical with those in pGL-AN [28,29] used in this study.

2.2. Expression vectors

Vector pME18Neo-Fvpr was used to express HIV-1 Vpr with a FLAG tag at the N-terminus [30]. Expression vectors for HIV-2 Vpr and Vpx with FLAG tags designated pME18Neo-Fvpr2 and pME18Neo-Fvpx were constructed by replacement of the *vpr* of pME18Neo-Fvpr with *vpr* and *vpx*, respectively. *Vpr/vpx* were amplified by polymerase chain reaction (PCR) using pGL-AN [28,29] as a template. Vectors, a pGL3-Control Vector (Promega, Madison, WI, USA), pSG-Vif cFLAG [31], and pSG-Gag (p24) cFLAG [32] were used to express luciferase, HIV-1 Vif and HIV-1 Gag-p24, respectively. These vectors were transiently transfected into 293T cells [33] by the calcium phosphate coprecipitation method as previously described [34]. 293T cells were cultured in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) [33].

2.3. Full-length viral clones

For construction of full-length viral clones with tags at the 5′ site of *vpx/vpr* designated pGL-xFrH and pGL-xHrF, the *Xba* I-*Eco*R I fragment of pGL-AN [28,29] (nucleotides 5064-5756) was cloned into pUC19 to construct a subcloning vector pUC-GL(Xb-Ec). The FLAG and HA sequences were then introduced right after the ATG codon of *vpx/vpr* genes in pUC-GL(Xb-Ec) by a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), and the resultant DNA fragment was cloned back into pGL-AN [28,29] to construct pGL-xFrH and pGL-xHrF, as shown in the text. These clones were transiently transfected into 293T cells [33] by the calcium phosphate coprecipitation method as above [34].

2.4. Establishment of HeLa Tet-Off cell lines

Expression vectors based on pBI-EGFP under the control of an inducible and bidirectional promoter (Clontech, Palo Alto, CA, USA) were used to express EGFP and HIV Vpr/Vpx simultaneously. Vectors designated pBI-EGFP/Vpr, pBI-EGFP/Vpr2 and pBI-EGFP/Vpx to express HIV-1 Vpr, HIV-2 Vpr, and HIV-2 Vpx with a FLAG tag at N-terminus, respectively, were constructed by insertion of *vpr/vpx* and FLAG sequences into pBI-EGFP. HIV-1 *vpr* and HIV-2 *vpr/vpx* were amplified by PCR using pNL432 (GenBank accession no. AF324493) [34] and pGL-AN [28,29] as templates, respectively. Transient transfection into HeLa Tet-Off cells, cultured as described in the Tet Systems User Manual, was performed by the calcium phosphate coprecipitation method [34]. For establishment of Tet-Off/control and Tet-Off/Vpx, HeLa Tet-Off cells were transfected with pBI-EGFP or pBI-EGFP/Vpx as described above [34], and cultured in the selection medium described in the manual. These cell lines were maintained in Eagle's minimal essential medium supplemented with 10% heat-inactivated FBS in the presence of G418 (0.1 mg/ml), hygromycin B (0.1 mg/ml) and doxycycline (0.1 μg/ml). Induction of Vpr/Vpx and EGFP from these cells was achieved by removal of doxycycline from the culture medium.

2.5. Luciferase assay

Luciferase assays were performed with a Luciferase Assay System (Promega, Madison, WI, USA).

2.6. Western immunoblotting

Western immunoblotting was performed essentially as previously described [35]. Cell lysates for immunoblotting were prepared from 293T and HeLa Tet-Off cells transfected with various clones by CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)/DOC (deoxycholate) [35] or Laemmli's sample [32] buffer, and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to polyvinylidene fluoride membranes, and the membranes were treated with an ANTI-FLAG M2 Monoclonal Antibody (Ab) (Sigma–Aldrich, St. Louis, MO, USA), anti-EGFP Ab (Living Colors A.v. Peptide Ab, BD Biosciences, Palo Alto, CA, USA) or anti-HA Ab (Monoclonal Ab, HA.11, BAbCO, Berkeley, CA, USA). For visualization, ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, England) were used.

3. Results and discussion

3.1. Structure of HIV Vpr/Vpx

We made a structural comparison of HIV-1 Vpr and HIV-2 Vpr/Vpx by generating sequence alignment through the Clustal W [23] program. As shown in Fig. 1A, the three proteins have three major α-helices with amphipathic characteristics surrounded by N- and C-terminal loops.

Sequence homologies of HIV-1 Vpr vs. HIV-2 Vpr, HIV-1 Vpr vs. HIV-2 Vpx, and HIV-2 Vpr vs. HIV-2 Vpx are 44%, 24% and 22%, respectively. These figures show that sequence of HIV-2 Vpx is quite distinct from those of the other two. Sequence identities of HIV-2 Vpx with HIV-1 Vpr and HIV-2 Vpr are particularly low in a major helix 2 (nos. 47–55) and a region between major helices 2 and 3 (nos. 56–72). In this region (nos. 56–72), HIV-2 Vpx uniquely contains a short α-helix and a long loop. Tyrosines (nos. 68, 71 and 73) in and close to this region of Vpx have been reported to be critical for virion incorporation [36]. Furthermore, in the first half of the C-terminal loop (nos. 93–105), HIV-2 Vpx characteristically bears several glycines. Instead of the glycines, there are a group of hydrophilic amino acids and arginines in the corresponding regions of the two Vpr (nos. 95–104). The two arginines (nos. 100 and 101) of HIV-1 Vpr have been reported to be responsible for G_2 arrest [37].

Vpr/Vpx of HIV-2 have different characteristics from HIV-1 Vpr in N- and C-terminal loops. N-terminal loops of HIV-2 Vpr/Vpx are longer than that of HIV-1 Vpr, and there are two prolines in the region (nos. 8–14). In the second half of the C-terminal loop of HIV-2 Vpx, there are continuous seven prolines (nos. 106–112). HIV-2 Vpr similarly has several prolines (nos.102, 104, 107 and 109), while HIV-1 Vpr has no prolines in its C-terminal loops.

Sequence identities of HIV-2 Vpr/Vpx with HIV-1 Vpr are enough to build an appropriate homology model for HIV-

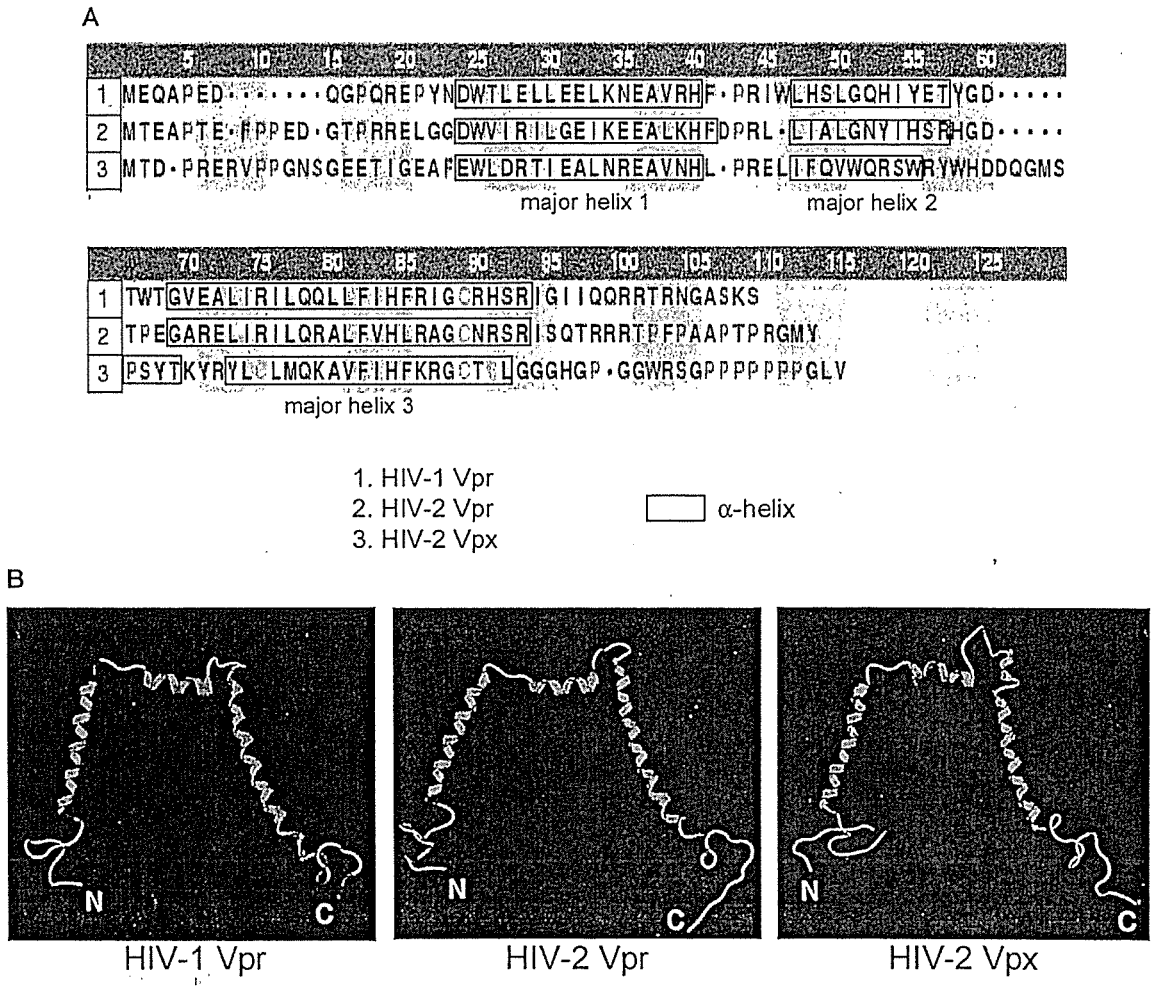

Fig. 1. Structure of HIV Vpr/Vpx proteins. (A) Sequence alignment of Vpr/Vpx. The sequence alignment, was performed, by the Clustal W program [23]. Colored codes indicate amino acids with distinct characteristics as follows: red, acidic; blue, basic; pink, hydrophilic; green, hydrophobic; light green, aromatic; yellow, cysteines; orange, prolines. Amino acids that constitute α-helices in HIV-1 Vpr as determined by NMR [9], or are predicted to constitute α-helices in HIV-2 Vpr/Vpx, are boxed. Amino acid sequence of HIV-1 Vpr and those of HIV-2 Vpr/Vpx are from HIV-1 P896 (GenBank accession no. U39362) and HIV-2 GH-1 (GenBank accession no. M30895) isolates, respectively. (B) Ribbon diagram of Vpr/Vpx. On the basis of the NMR structure of HIV-1 Vpr (PDB code 1ESX) [9], the 3-D structure of HIV-2 Vpr/Vpx was predicted by homology modeling from the sequence alignment shown in (A). Colored parts of the diagram indicate unique structures as follows: red, α-helices; blue, tight turn (3_{10} helices); green, other turn; light blue, others. N and C indicate N- and C-termini of the proteins, respectively.

2 Vpr/Vpx based on the NMR structure of HIV-1 Vpr [9]. 3-D structures of HIV-2 Vpr/Vpx were thus predicted by the MODELLER 6v2 program [24], and their diagrams were generated by RASMOL software [25]. As is clear in Fig. 1B, the three proteins share a basic framework, but there are unique structural features in the N-terminal loop, the region between major helices 2 and 3, and the C-terminal loop of HIV-2 Vpx. The different shape of Vpx in these regions may be associated with its conspicuous activity, e.g. lack of ability to induce G_2 arrest [13,14,18,19,21].

3.2. Cytopathogenic activity of HIV Vpr/Vpx

HIV Vpr has been shown to have cytopathogenic activities such as induction of G_2 arrest and apoptosis [10–21], while there have been no reports to demonstrate Vpx having this activity [13,14,18,19,21]. The cytopathogenic potential of Vpx was assessed by transfection experiments using the two Vprs as controls. We first monitored the production of marker protein luciferase upon co-expression. 293T cells were transiently co-transfected with expression vectors of luciferase and Vpr/Vpx, and cell lysates were analyzed for luciferase activity. As shown in Fig. 2A, when HIV-1 Vif and Gag-p24 were co-expressed, production of luciferase was not significantly affected. In contrast, when HIV Vpr/Vpx were co-expressed, production of luciferase was markedly decreased. To normalize the detrimental activity of the Vpr/Vpx observed here, the expression level of HIV Vpr/Vpx proteins in the cell lysates was determined by Western blot analysis. As shown in Fig. 2B, the expression level of HIV-2 Vpr was much lower than that of HIV-1 Vpr and HIV-2 Vpx. Taken together, the cytopathogenic activity of HIV-2 Vpr was much higher than those of HIV-1 Vpr and HIV-2 Vpx.

To evaluate more definitively the detrimental effects of Vpr/Vpx on cells, we next established various HeLa-Tet Off cell lines, which carry an inducible promoter for expression of the two proteins. Expression vectors of Vpr/Vpx designated pBI-EGFP/Vpr, pBI-EGFP/Vpr2 and pBI-EGFP/Vpx were constructed, and transfected into HeLa-Tet Off cells. The cells were then incubated in the absence (induction +) or presence (induction –) of doxycycline, and cell lysates were prepared. As shown in Fig. 3A, while expression of marker proteins EGFP, HIV-1 Vpr, and HIV-2 Vpx was readily observed upon induction, HIV-2 Vpr was not detected. Stable HeLa-Tet Off cell lines carrying pBI-EGFP (Tet-Off/control) and pBI-EGFP/Vpx (Tet-Off/Vpx) were then established. Cell lines harboring HIV-1 *vpr* and HIV-2 *vpr* genes were found to be unstable and difficult to maintain. A tiny amount of cytotoxic Vpr could cause the death of target cells. As expected, expression of HIV-2 Vpx in Tet-Off/Vpx cells was induced by removal of doxycycline (Fig. 3B). Using these cell lines, the effect of Vpx on cells was monitored by determining cell numbers. As shown in Fig. 3C, Tet-Off/Vpx cells did not grow substantially after induction of Vpx. In sharp contrast, Tet-Off/control cells and Tet-Off/Vpx cells without induction grew

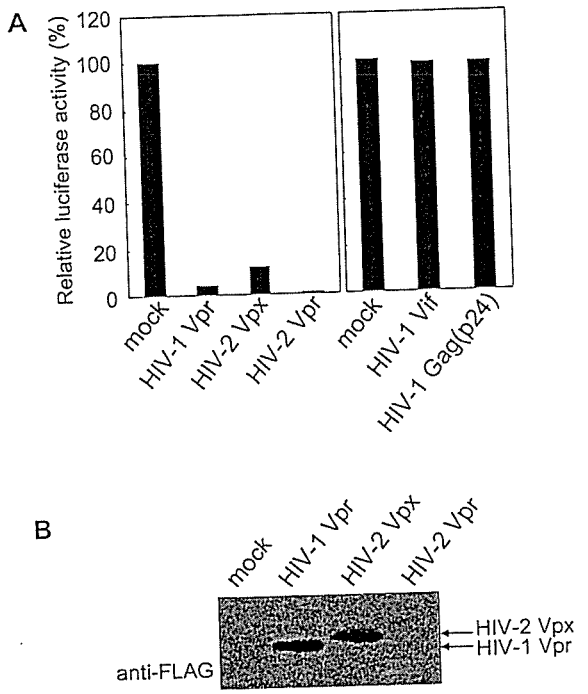

Fig. 2. Expression of HIV Vpr/Vpx in 293T cells. (A) Effects in cells of Vpr/Vpx on luciferase production. 293T cells were co-transfected with the expression vector for luciferase (pGL3-Control Vector) (2.5 μg), and one of the expression vectors for Vpr/Vpx (pME18Neo-Fvpr [30], pME18Neo-Fvpx and pME18Neo-Fvpr2) (7.5 μg) (left panel) or of those for Vif/Gag-p24 (pSG-Vif cFLAG [31] and pSG-Gag (p24) cFLAG [32]) (7.5 μg) (right panel). At 48 h post-transfection, cells were harvested for luciferase assays (A) and for Western blotting analysis (B). Luciferase activity relative to that of mock (pUC19 in the left panel and pSG5 in the right panel)-transfected cells is shown. (B) Expression level of Vpr/Vpx as monitored by Western blot analysis. Cell lysates were analyzed by Western immunoblotting using anti-FLAG Ab (anti-FLAG M2 monoclonal Ab). Loading amount in each lane was normalized by the luciferase activity shown in (A). Mock, pUC19.

fairly well. These results clearly demonstrated for the first time, the cytostatic effect of HIV-2 Vpx. We were interested in the extremely low expression level of HIV-2 Vpr observed in Fig. 2B and Fig. 3A. Expression of Vpr in the context of the full-length HIV-2 genome was, therefore, examined. For this purpose, FLAG and HA tags were introduced into the 5′-ends of *vpx* and *vpr* genes of pGL-AN in different combinations (Fig. 4A). The resultant clones were transfected into 293T cells, and cell lysates were analyzed by Western blotting using anti-FLAG and anti-HA Abs. As shown in Fig. 4B, Vpx was easily detected in cells transfected with pGL-xFrH and pGL-xHrF. In contrast, expression of Vpr was not observed at all in the transfected cells. Proteasome degradation may account for the extremely low expression level of HIV-2 Vpr observed here, as reported for SIVmac [38].

3.3. Perspectives relating to this study

We used the NMR structure of HIV-1 Vpr analyzed in H_2O/trifluoroetanol as a template [9] in the homology modeling here. When acetonitrile was used instead of trifluoroethanol, folding of three α-helices around a hydrophobic core,

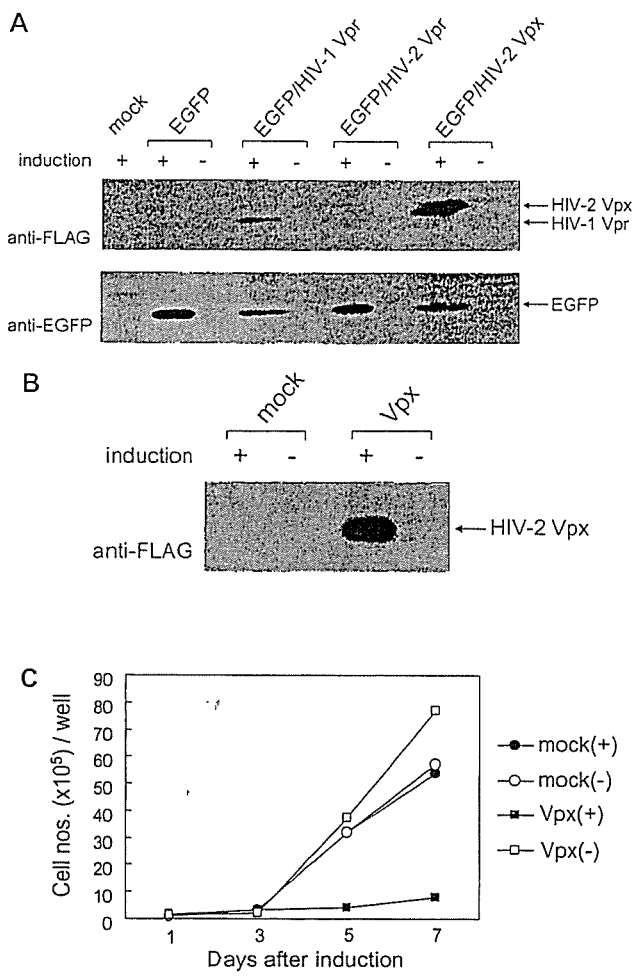

Fig. 3. Expression of HIV Vpr/Vpx in HeLa Tet-Off cells. (A) Transient expression of Vpr/Vpx in HeLa Tet-Off cells. Cells were transfected with various expression vectors (pBI-EGFP, pBI-EGFP/Vpr, pBI-EGFP/Vpr2 and pBI-EGFP/Vpx) (15 μg), and incubated for 48 h with (+) or without (–) induction. Cell lysates were then prepared and analyzed by Western immunoblotting using anti-FLAG Ab (ANTI-FLAG M2 Monoclonal Ab, upper) and anti-EGFP Ab (Living Colors A.v. Peptide Ab, lower). Induction of Vpr/Vpx and EGFP was done by removal of doxycycline from the culture medium. Mock, pUC19. (B) Expression of Vpx in HeLa Tet-Off cells stably carrying pBI-EGFP/Vpx (Tet-Off/Vpx). Cells were incubated for 5 days with (+) or without (–) induction, and lysates were prepared for Western immunoblotting using anti-FLAG Ab (ANTI-FLAG M2 Monoclonal Ab). Each lane contained 20 μg of total protein. As a control, HeLa Tet-Off cells carrying pBI-EGFP (Tet-Off/control) were used. (C) Growth of Tet-Off/control and Tet-Off/Vpx cells. Cells were cultured for 7 days with (+) or without (–) induction, and nos. of cells were determined at intervals. Variable cell counts and days after induction are plotted.

which is more likely to be formed in physiological conditions, was observed [39]. Homology modeling based on this structure should be carried out.

Fletcher et al. suggested that two major functions carried out by HIV-1 Vpr, G_2 arrest and nuclear import of viral reverse transcription complexes, are borne by Vpr and Vpx of SIVsm, respectively [18]. In this study, however, we found that Vpx has cytopathogenic activity, the same as Vpr. We need to clarify the structural basis for this activity.

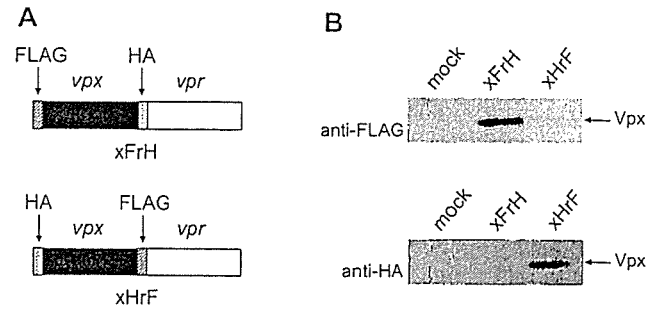

Fig. 4. Expression of Vpr/Vpx in the context of the full-length HIV-2 genome. (A) Location of tags in pGL-xFrH and pGL-xHrF. FLAG and HA tags are placed in the *vpx/vpr* region as indicated. (B) Expression level in cells of Vpx/Vpr. 293T cells were co-transfected with one of the full-length viral clones (pGL-xFrH and pGL-xHrF) (7.5 μg) and the expression vector for luciferase (pGL3-Control Vector) (2.5 μg). At 48 h post-transfection, cells were harvested for luciferase assays and Western blot analysis using anti-FLAG Ab (ANTI-FLAG M2 Monoclonal Ab, upper) and anti-HA Ab (Monoclonal Ab, HA.11, lower). The loading amount in each lane was normalized by luciferase activity. Mock, pUC19.

Acknowledgements

We thank Ms. Kazuko Yoshida for editorial assistance. This work was supported by a Grant-in-Aid for Scientific Research (B) (14370103) from the Japan Society for the Promotion of Science, and a Grant-in-Aid for Scientific Research on Priority Areas (2) (16017270) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. B.K. is supported by a Royal Thai Government Scholarship 2003 (Commission on Higher Education), Thailand.

References

[1] V. Courgnaud, B. Abela, X. Pourrut, E. Mpoudi-Ngole, S. Loul, E. Delaporte, M. Peeters, Identification of a new simian immunodeficiency virus lineage with a *vpu* gene present among different *Cercopithecus* monkeys (*C. mona, C. cephus*, and *C. nictitans*) from Cameroon, J. Virol. 77 (2003) 12523–12534.

[2] M. Tristem, A. Purvis, D.L. Quicke, Complex evolutionary history of primate lentiviral *vpr* genes, Virology 240 (1998) 232–237.

[3] S. Souquiere, F. Bibollet-Ruche, D.L. Robertson, M. Makuwa, C. Apetrei, R. Onanga, C. Kornfeld, J.C. Plantier, F. Gao, K. Abernethy, L.J. White, W. Karesh, P. Telfer, E.J. Wickings, P. Mauclere, P.A. Marx, F. Barre-Sinoussi, B.H. Hahn, M.C. Muller-Trutwin, F. Simon, Wild *Mandrillus sphinx* are carriers of two types of lentivirus, J. Vorol. 75 (2001) 7086–7096.

[4] B.E. Beer, B.T. Foley, C.L. Kuiken, Z. Tooze, R.M. Goeken, C.R. Brown, J. Hu, M. St Claire, B.T. Korber, V.M. Hirsch, Characterization of novel simian immunodeficiency viruses from red-capped mangabeys from Niagala (SIVrcmNG409 and SIVrcmNG411), J. Virol. 75 (2001) 12014–12027.

[5] J. Hu, W.M. Switzer, B.T. Foley, D.L. Robertson, R.M. Goeken, B.T. Korber, V.M. Hirsch, B.E. Beer, Characterization and comparison of recombinant simian immunodeficiency virus from drill (*Mandrillus leucophaeus*) and mandrill (*Mandrillus sphinx*) isolates, J. Virol. 77 (2003) 4867–4880.

[6] S.M. Lang, M. Weeger, C. Stahl-Hennig, C. Coulibaly, G. Hunsmann, J. Muller, H. Muller-Hermelink, D. Fuchs, H. Wachter, M.M. Daniel, R.C. Desrosiers, B. Fleckenstein, Importance of *vpr* for infection of

rhesus monkeys with simian immunodeficiency virus, J. Virol. 67 (1993) 902–912.
[7] J.S. Gibbs, A.A. Lackner, S.M. Lang, M.A. Simon, P.K. Sehgal, M.D. Daniel, R.C. Desrosiers, Progression to AIDS in the absence of a gene for *vpr* or *vpx*, J. Virol. 69 (1995) 2378–2383.
[8] R.C. Desrosiers, J.D. Lifson, J.S. Gibbs, S.C. Czajak, A.Y.M. Howe, L.O. Arthur, R.P. Johnson, Identification of highly attenuated mutants of simian immunodeficiency virus, J. Virol. 72 (1998) 1431–1437.
[9] K. Wecker, N. Morellet, S. Bouaziz, B.P. Roques, NMR structure of the HIV-1 regulatory protein Vpr in H_2O/trifluoroethanol. Comparison with the Vpr N-terminal (1–51) and C-terminal (52–96) domains, Eur. J. Biochem. 269 (2002) 3779–3788.
[10] M.E. Rogel, L.I. Wu, M. Emerman, The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection, J. Virol. 69 (1995) 882–888.
[11] J.B. Jowett, V. Planelles, B. Poon, N.P. Shah, M.L. Chen, I.S. Chen, The human immunodeficiency virus type 1 *vpr* gene arrests infected T cells in the G_2 + M phase of the cell cycle, J. Virol. 69 (1995) 6304–6313.
[12] J. He, S. Choe, R. Walker, P. Di Marzio, D.O. Morgan, N.R. Landau, Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G_2 phase of the cell cycle by inhibiting p34cdc2 activity, J. Virol. 69 (1995) 6705–6711.
[13] F. Re, D. Braaten, E.K. Franke, J. Luban, Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G_2 by inhibiting the activation of $p34^{cdc2}$-cyclin B, J. Virol. 69 (1995) 6859–6864.
[14] P. Di Marzio, S. Choe, M. Ebright, R. Knoblauch, N.R. Landau, Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr, J. Virol. 69 (1995) 7909–7916.
[15] T. Fukumori, H. Akari, A. Yoshida, M. Fujita, A.H. Koyama, S. Kagawa, A. Adachi, Regulation of cell cycle and apoptosis by human immunodeficiency virus type 1 Vpr, Microbes Infect. 2 (2000) 1011–1017.
[16] S.A. Stewart, B. Poon, J.B. Jowett, I.S. Chen, Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest, J. Virol. 71 (1997) 5579–5592.
[17] V. Ayyavoo, A. Mahboubi, S. Mahalingam, R. Ramalingam, S. Kudchodkar, W.V. Williams, D.R. Green, D.B. Weiner, HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B, Nat. Med. 3 (1997) 1117–1123.
[18] T.M. Fletcher III, B. Brichacek, N. Sharova, M.A. Newman, G. Stivahtis, P.M. Sharp, M. Emerman, B.H. Hahn, M. Stevenson, Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV_{SM}, EMBO J. 15 (1996) 6155–6165.
[19] V. Planelles, J.B. Jowett, Q.X. Li, Y. Xie, B. Hahn, I.S. Chen, Vpr-induced cell cycle arrest is conserved among primate lentiviruses, J. Virol. 70 (1996) 2516–2524.
[20] G.L. Stivahtis, M.A. Soares, M.A. Vodicka, B.H. Hahn, M. Emerman, Conservation and host specificity of Vpr-mediated cell cycle arrest suggest a fundamental role in primate lentivirus evolution and biology, J. Virol. 71 (1997) 4331–4338.
[21] S.M. Mueller, S.M. Lang, The first HxRxG motif in simian immunodeficiency virus mac239 Vpr is crucial for G_2/M cell cycle arrest, J. Virol. 76 (2002) 11704–11709.
[22] Y. Zhu, H.A. Gelbard, M. Roshal, S. Pursell, B.D. Jamieson, V. Planelles, Comparison of cell cycle arrest, transactivation, and apoptosis induced by the simian immunodeficiency virus SIVagm and human immunodeficiency virus type 1 *vpr* genes, J. Virol. 75 (2001) 3791–3801.
[23] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[24] A. Sali, T.L. Blundell, Comparative protein modelling by satisfaction of spatial restrains, J. Mol. Biol. 234 (1993) 779–815.
[25] R.A. Sayle, E.J. Milner-White, RASMOL: biomolecular graphics for all, Trends Biochem. Sci. 20 (1995) 374.
[26] F.M. Kim, D.L. Kolson, J.W. Balliet, A. Srinivasan, R.G. Collman, V3-independent determinants of macrophage tropism in a primary human immunodeficiency virus type 1 isolate, J. Virol. 69 (1995) 1755–1761.
[27] A. Hasegawa, H. Tsujimoto, N. Maki, K.-I. Ishikawa, T. Miura, M. Fukasawa, K. Miki, M. Hayami, Genomic divergence of HIV-2 from Ghana, AIDS Res. Hum. Retroviruses 5 (1989) 593–604.
[28] M. Kawamura, H. Sakai, A. Adachi, Human immunodeficiency virus Vpx is required for the early phase of replication in peripheral blood mononuclear cells, Microbiol. Immunol. 38 (1994) 871–878.
[29] F. Ueno, H. Shiota, M. Miyaura, A. Yoshida, A. Sakurai, J. Tatsuki, A.H. Koyama, H. Akari, A. Adachi, M. Fujita, Vpx and Vpr proteins of HIV-2 up-regulate the viral infectivity by a distinct mechanism in lymphocytic cells, Microbes Infect. 5 (2003) 387–395.
[30] Y. Nishino, T. Myojin, M. Kamata, Y. Aida, Human immunodeficiency virus type 1 Vpr gene product prevents cell proliferation on mouse NIH3T3 cells without the G2 arrest of the cell cycle, Biochem. Biophys. Res. Commun. 232 (1997) 550–554.
[31] H. Wang, A. Sakurai, B. Khamsri, T. Uchiyama, H. Gu, A. Adachi, M. Fujita, Unique characteristics of HIV-1 Vif expression, Microbes Infect. 7 (2005) 385–390.
[32] M. Fujita, H. Akari, A. Sakurai, A. Yoshida, T. Chiba, K. Tanaka, K. Strebel, A. Adachi, Expression of HIV-1 accessory protein Vif is controlled uniquely to be low and optimal by proteasome degradation, Microbes Infect. 6 (2004) 791–798.
[33] J.S. Lebkowski, S. Clancy, M.P. Calos, Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression, Nature 317 (1985) 169–171.
[34] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.
[35] R.L. Willey, D.H. Smith, L.A. Lasky, T.S. Theodore, P.L. Earl, B. Moss, D.J. Capon, M.A. Martin, In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity, J. Virol. 62 (1988) 139–147.
[36] S. Mahalingam, B.V. Tine, M.L. Santiago, F. Gao, G.M. Shaw, B.H. Hahn, Functional analysis of the simian immunodeficiency virus Vpx protein: identification of packaging determinants and a novel nuclear targeting domain, J. Virol. 75 (2001) 362–374.
[37] S. Iijima, Y. Nitahara-kasahara, K. Kimata, W.Z. Zhuang, M. Kamata, M. Isogai, M. Miwa, Y. Tsunetsugu-Yokota, Y. Aida, Nuclear localization of Vpr is crucial for the efficient replication of HIV-1 in primary $CD4^+$ T cells, Virology 327 (2004) 249–261.
[38] M.A. Accola, A.A. Bukovsky, M.S. Jones, H.G. Gottlinger, A conserved dileucine-containing motif in $p6^{gag}$ governs the particle association of Vpx and Vpr of simian immunodeficiency viruses SIV_{mac} and SIV_{agm}, J. Virol. 73 (1999) 9992–9999.
[39] N. Morellet, S. Bouaziz, P. Petitjean, B.P. Roques, NMR structure of the HIV-1 regulatory protein VPR, J. Mol. Biol. 327 (2003) 215–227.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Microbes and Infection 8 (2006) 1075–1081
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Construction of *gag*-chimeric viruses between HIV-1 and SIVmac that are capable of productive multi-cycle infection

Kazuya Kamada, Akiko Yoshida, Boonruang Khamsri, Ahmad Piroozmand, Tomoki Yamashita, Tsuneo Uchiyama, Mikako Fujita*, Akio Adachi

Department of Virology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima-shi, Tokushima 770-8503, Japan

Received 12 October 2005; accepted 4 November 2005
Available online 19 January 2006

Abstract

Forty-nine recombinant viral clones between human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus from the rhesus monkey (SIVmac), which carry chimeric *gag* (capsid/p2 region) genes in the background of the HIV-1 genome, were constructed to establish an HIV-1/monkey infection model system for human AIDS. Upon transfection, all the recombinants generated progeny virions at a level comparable to the parental HIV-1 clone and no major abnormalities were found in the virions, as examined by Western blot analysis. In infection experiments, 18 recombinants grew in human lymphocytic cells and six of these clones propagated as well as the parental virus, as monitored by virion associated-reverse transcriptase production. By contrast, none of the recombinants grew at a detectable level in monkey lymphocytic cells. The defective replication site(s) in human cells for non-infectious recombinants was mapped to the step before and/or during reverse transcription. Our results described here showed that HIV-1 type chimeric viruses between HIV-1 and SIVmac, which are capable of spreading productive infection, are readily constructed throughout the capsid/p2 region. In addition, it is suggested that there may be a viral determinant(s), other than Gag, responsible for the species-specific tropism of HIV-1 and which is associated with viral DNA synthesis.

Keywords: HIV-1; SIVmac; Gag; Capsid/p2; Chimeric virus

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) has been shown to have a much narrower host range than simian immunodeficiency viruses (SIVs), such as SIVmac [1]. This species-specific tropism of HIV-1 (tropism for humans and chimpanzees) has hindered the development of effective model systems for basic AIDS study. Early works have demonstrated that the non-*env* sequence is critical for the species tropism [2,3]. While SIVmac grows well both in human and simian lymphocytes, HIV-1 does not replicate in the latter cells, and the major viral determinant(s) for this restriction is most likely to be the Gag capsid (CA)-p2 region of HIV-1 [2,4–7]. Furthermore, mutations in *gag* can affect the cellular tropism of HIV-1. Some *gag* mutant viruses, with a postentry early defect in some human lymphocytic cells, were shown to grow well in others [8–11]. On the basis of these studies, it is quite likely that the early function of Gag, i.e., uncoating and/or reverse transcription, is involved in the restriction of HIV-1 growth in monkey cells. By extensive genetic and molecular analyses, recent studies have clearly indicated that Gag-CA is associated with the postentry early replication block of HIV-1 in monkey cells [12–14].

To develop a new and effective model of HIV-1 infection in practically useful non-human primates, recombinant viruses between HIV-1 and SIVmac in an HIV-1 background are critically required. In this report, various sequences in the SIVmac CA-spacer domain were inserted into the corresponding regions of HIV-1 to generate HIV-1-based *gag*-chimeric viruses. Forty-nine recombinants thus constructed were examined for their ability to grow in human and simian lymphocytic cell

* Corresponding author. Tel.: +81 88 633 9232; fax: +81 88 633 7080.
E-mail address: mfujita@basic.med.tokushima-u.ac.jp (M. Fujita).

1286-4579/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.micinf.2005.11.006

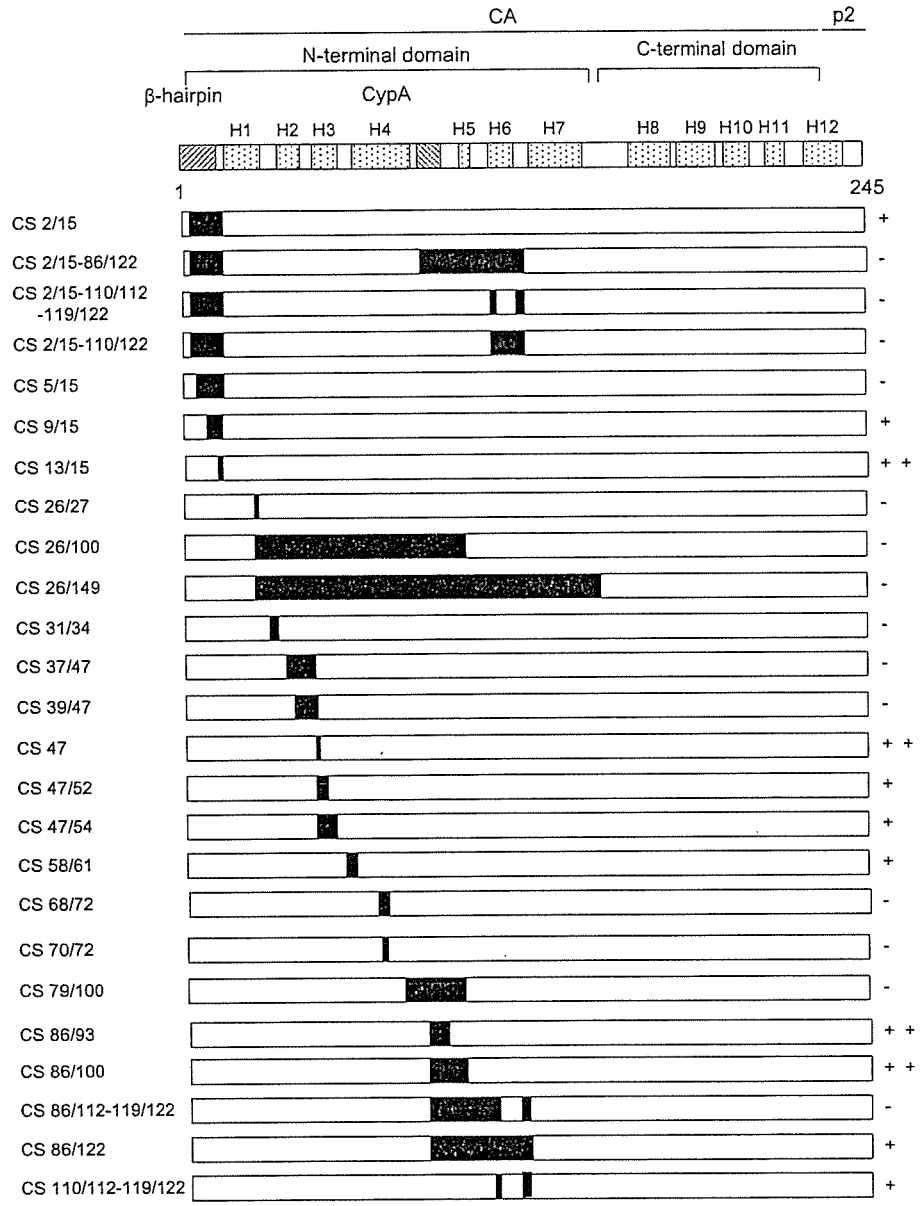

Fig. 1. *Gag*-chimeric viruses between HIV-1 and SIVmac used in this study. Location of SIVmac Gag sequence (MA239) inserted into HIV-1 Gag CA-p2 region (NL432) is indicated by black area. For the sequences inserted, see Table 1. Growth ability of viruses in M8166 cells is given as ++ (wt growth), + (retarded growth), and − (no growth) on the right. For examples of virus growth kinetics, see Fig. 2. Structural domains of HIV-1 Gag CA-p2 [29,30] are indicated at the top. H, α-helix; Cyp A, cyclophilin A-binding loop.

subjected to Western blot analysis with a human anti-HIV-1 antiserum as reported previously [19].

2.5. Polymerase chain reaction (PCR) analysis

M8166 cells were infected with an equal amount of cell-free virus samples from transfected 293T cells for 16 h in the presence of EGTA/DNase I [11,27]. On day 2 post-infection, cells were harvested for DNA extraction as previously described [27]. To monitor viral DNA synthesis in cells, DNA samples were PCR-amplified and analyzed essentially as previously described [27]. For the amplification of viral DNA, the early (R/U5) and late (U5/5′-non-coding region) primer pairs [27] were used. As a control for PCR, β-globin was amplified as previously described [16,28].

3. Results

3.1. Construction and biological characterization of gag-chimeric clones

We have recently shown that the transfer of a minute region of SIVmac CA to the corresponding region of HIV-1 could confer the cyclophilin A-independent replication potential of

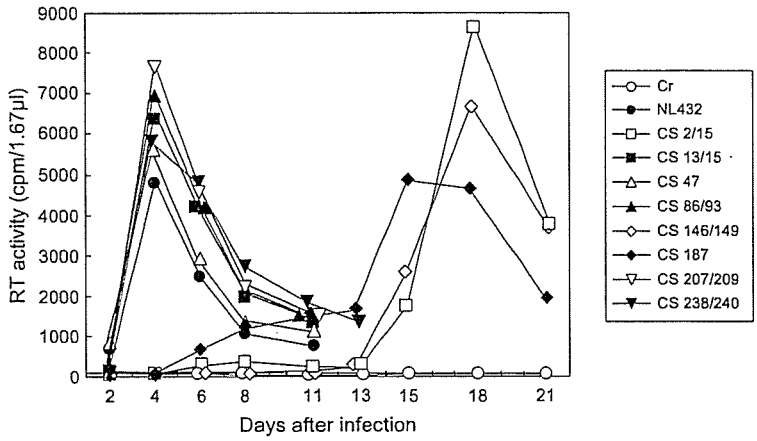

Fig. 2. Growth kinetics in M8166 cells of chimeric viruses. Cells were infected with cell-free viruses as described in the text, and virus replication was monitored at intervals by RT production in the culture supernatants. Input viruses were prepared from 293T cells transfected with 20 μg of the clones indicated on the right. Cr, pUC19.

almost all recombinants carrying an insertion in the α-helix grew poorly or not at all (Fig. 1). We then inoculated all the recombinants (1×10^7 RT units for each) into monkey lymphocytic HSC-F cells (1×10^7 cells) using SIVmac prepared from 293T cells transfected with pMA239 [2] as a positive control. No recombinants were found to be infectious for HSC-F cells (data not shown).

3.2. Biochemical characterization of gag-chimeric clones

Fourteen recombinants were selected and examined for their biochemical properties in cells. These included non-infectious (CS5/15, CS39/47, CS86/112-119/122, CS153/154, CS204 and CS235/245-f), poorly infectious (CS9/15 and CS146/149) and highly infectious (CS13/15, CS47, CS86/93, CS86/100, CS207/209 and CS238/240) clones for M8166 cells. The insertion sites of SIVmac *gag* sequences in these recombinants are located throughout the CA-p2 region of HIV-1 (Fig. 1).

First, the Gag expression in cells and the Gag profile in virions were confirmed. Because RT production in 293T cells transfected with the recombinants was fairly normal, no major defects were expected to be observed. 293T cells were transfected with various clones, and on day 2 post-transfection, cells were harvested for Western blot analysis. As shown in Fig. 3, no clear abnormality was seen for the recombinants tested, except CS235/245-f. Consistent with the insertion

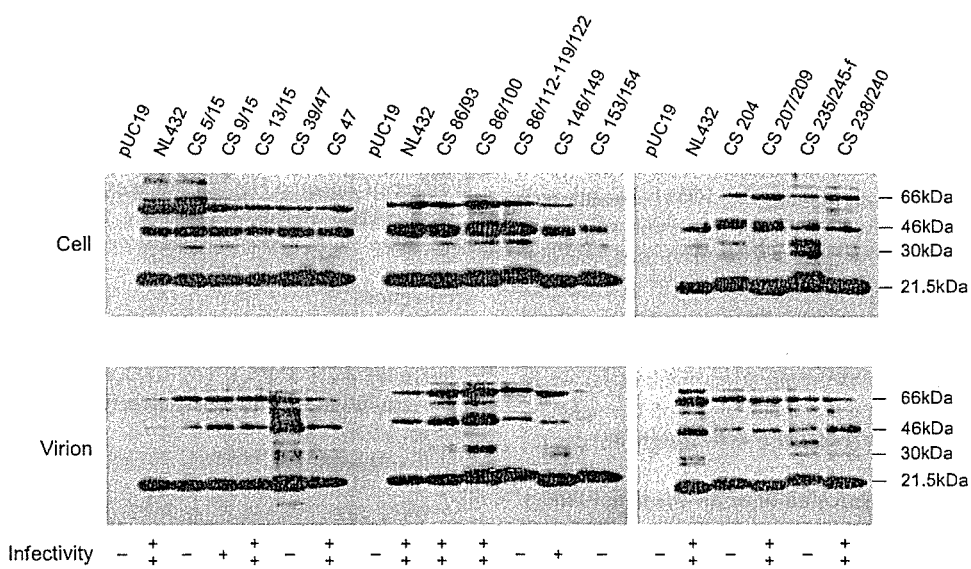

Fig. 3. Western blot analysis of chimeric viruses. Cell and virion lysates were prepared from 293T cells transfected with various clones (20 μg) indicated at the top, as described previously [20,25,26]. Each sample was then subjected to Western blot analysis using a human anti-HIV-1 antiserum as reported before [19]. Results obtained from three independent experiments are shown. The infectivity of viruses for M8166 cells is given as ++ (wt growth), + (retarded growth) and − (no growth) at the bottom. Protein size is shown on the right. Cell, lysates from transfected cells; virion, virion lysates.

[5] R. Shibata, H. Sakai, M. Kawamura, K. Tokunaga, A. Adachi, Early replication block of human immunodeficiency virus type 1 in monkey cells, J. Gen. Virol. 76 (1995) 2723–2730.
[6] K. Uberla, C. Stahl-Henning, D. Bottiger, K. Matz-Rensing, F.J. Kaup, J. Li, W.A. Haseltine, B. Fleckenstein, G. Hunsmann, B. Oberg, Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 8210–8214.
[7] T. Dorfman, H.G. Gottlinger, The human immunodeficiency virus type 1 capsid p2 domain confers sensitivity to the cyclophilin-binding drug SDZ NIM811, J. Virol. 70 (1996) 5751–5757.
[8] J. Sakuragi, H. Sakai, M. Kawamura, K. Tokunaga, S. Ueda, A. Adachi, Generation and characterization of a host cell-dependent *gag* gene mutant of human immunodeficiency virus type 1, Virology 212 (1995) 251–254.
[9] R.A. Furuta, R. Shimano, T. Ogasawara, R. Inubushi, K. Amano, H. Akari, M. Hatanaka, M. Kawamura, A. Adachi, HIV-1 capsid mutants inhibit the replication of wild-type virus at both early and late infection phase, FEBS Lett. 415 (1997) 231–234.
[10] M. Kawamura, R. Shimano, R. Inubushi, H. Akari, A. Adachi, Early function of HIV-1 Gag proteins is cell-dependent, Biochem. Bioph. Res. Co. 248 (1998) 899–903.
[11] K.-B. Koh, M. Miyaura, A. Yoshida, A. Sakurai, M. Fujita, A. Adachi, Cell-dependent *gag* mutants of HIV-1 are crucially defective at the stage of uncoating/reverse transcription in non-permissive cells, Microbes Infect. 2 (2000) 1419–1423.
[12] C.M. Owens, B. Song, M.J. Perron, P.C. Yang, M. Stremlau, J. Sodroski, Binding and susceptibility to postentry restriction factors in monkey cells are specified by distinct regions of the human immunodeficiency virus type 1 capsid, J. Virol. 78 (2004) 5423–5437.
[13] T. Hatziioannou, S. Cowan, U.K. von Schwedler, W.I. Sundquist, P.D. Bieniasz, Species-specific tropism determinants in the human immunodeficiency virus type 1 capsid, J. Virol. 78 (2004) 6005–6012.
[14] Y. Ikeda, L.M.J. Ylinen, M. Kahar-Bador, G.J. Towers, Influence of *gag* on human immunodeficiency virus type 1 species-specific tropism, J. Virol. 78 (2004) 11816–11822.
[15] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.
[16] F. Ueno, H. Shiota, M. Miyaura, A. Yoshida, A. Sakurai, J. Tatsuki, A.H. Koyama, H. Akari, A. Adachi, M. Fujita, Vpx and Vpr proteins of HIV-2 up-regulate the viral infectivity by a distinct mechanism in lymphocytic cells, Microbes Infect. 5 (2003) 387–395.
[17] A. Adachi, N. Ono, H. Sakai, K. Ogawa, R. Shibata, T. Kiyomasu, H. Masuike, S. Ueda, Generation and characterization of the human immunodeficiency virus type 1 mutants, Arch. Virol. 117 (1991) 45–58.
[18] M. Fujita, A. Sakurai, A. Yoshida, M. Miyaura, A.H. Koyama, K. Sakai, A. Adachi, Amino acid residues 88 and 89 in the central hydrophilic region of human immunodeficiency virus type 1 Vif are critical for viral infectivity by enhancing the steady-state expression of Vif, J. Virol. 77 (2003) 1626–1632.
[19] M. Fujita, H. Akari, A. Sakurai, A. Yoshida, T. Chiba, K. Tanaka, K. Strebel, A. Adachi, Expression of HIV-1 accessory protein Vif is controlled uniquely to be low and optimal by proteasome degradation, Microbes Infect. 6 (2004) 791–798.
[20] M. Fujita, A. Yoshida, M. Miyaura, A. Sakurai, H. Akari, A.H. Koyama, A. Adachi, Cyclophilin A-independent replication of a human immunodeficiency virus type 1 isolate carrying a small portion of the simian immunodeficiency virus SIV_{MAC} *gag* capsid region, J. Virol. 75 (2001) 10527–10531.
[21] J.S. Lebkowski, S. Clancy, M.P. Calos, Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression, Nature 3 (1985) 169–171.
[22] H. Akari, T. Fukumori, S. Iida, A. Adachi, Induction of apoptosis in Herpesvirus saimiri-immortalized T lymphocytes by blocking interaction of CD28 with CD80/CD86, Biochem. Bioph. Res. Co. 263 (1999) 352–356.
[23] T. Folks, S. Benn, A. Rabson, T. Theodore, M.D. Hoggan, M. Martin, M. Lightfoote, K. Sell, Characterization of a continuous T-cell line susceptible to the cytopathic effects of acquired immune deficiency syndrome (AIDS)-associated retrovirus, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 4539–4543.
[24] R.L. Willey, D.H. Smith, L.A. Lasky, T.S. Theodore, P.L. Earl, B. Moss, D.J. Capon, M.A. Martin, In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity, J. Virol. 62 (1988) 139–147.
[25] H. Akari, S. Arold, T. Fukumori, T. Okazaki, K. Strebel, A. Adachi, Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation, J. Virol. 74 (2000) 2907–2912.
[26] H. Akari, T. Fukumori, A. Adachi, Cell-dependent requirement of human immunodeficiency virus type 1 gp41 cytoplasmic tail for Env incorporation into virions, J. Virol. 74 (2000) 4891–4893.
[27] K.-B. Koh, M. Fujita, A. Adachi, Elimination of HIV-1 plasmid DNA from virus samples obtained from transfection by calcium-phosphate co-precipitation, J. Virol. Methods 90 (2000) 99–102.
[28] H.M. Bauer, Y. Ting, C.S. Grier, J.C. Chambers, C.J. Tashiro, J. Chimera, A. Reingold, M.M. Manos, Genital human papilloma virus infection in female university students as determined by a PCR-based method, J. Am. Med. Assoc. 265 (1991) 472–477.
[29] T.R. Gamble, F.F. Vajdos, S. Yoo, D.K. Worthylake, M. Houseweart, W.I. Sundquist, C.P. Hill, Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid, Cell 87 (1996) 1285–1294.
[30] T.R. Gamble, S. Yoo, F.F. Vajdos, U.K. von Schwedler, D.K. Worthylake, H. Wang, J.P. McCutcheon, W.I. Sundquist, C.P. Hill, Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein, Science 278 (1997) 849–853.
[31] H. Akari, M. Fujita, S. Kao, M.A. Khan, M. Shehu-Xhilaga, A. Adachi, K. Strebel, High level expression of human immunodeficiency virus type-1 Vif inhibits viral infectivity by modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site, J. Biol. Chem. 279 (2004) 12355–12362.

神経系感染症が疑われたとき

ポイント

- 発熱を伴った頭蓋内圧亢進症状，脳実質障害があれば，髄膜炎，脳炎を積極的に疑うべきである．
- 食欲不振，倦怠感といった不定愁訴（結核性髄膜炎など）や精神症状（単純ヘルペス脳炎など）が発熱に先行する場合があり注意を要する．
- ときに，発熱が前景に立たないで性格変化や痴呆症状が中核をなす一群の神経感染症がある（スローウイルス感染症やプリオン病など）．
- 神経系感染症を疑う場合，髄液検査は病原診断へと導く第一歩である．ルーチンの検査でも病原体の種類を推察するにあたってある程度のところまで絞り込める．
- 一般的に画像検査（頭部 CT および MRI）や脳波検査では特異的な異常はないことが多いが，単純ヘルペス脳炎やプリオン病などではこれらの検査で特徴的な所見がみられることがある．

I どういう症状が出たら神経系感染症を疑うか？

神経系感染症は中枢神経系および末梢神経系感染症があり，症状はその障害部位に応じて多種多彩であるが，多くの場合発熱という炎症所見を伴った神経症状をみたら感染症を疑うべきである．

神経系感染症のうち，各種髄膜炎・脳炎が日常臨床で遭遇する最も頻度の高い疾患であるが頭痛，悪心，嘔吐といった頭蓋内圧亢進症状があれば髄膜炎を，加えて意識障害，失見当識・異常行動といった精神症状，

痙攣などの脳実質障害があれば，脳炎を積極的に疑うべきである．

- 食欲不振，倦怠感といった不定愁訴（結核性髄膜炎など）や精神症状（単純ヘルペス脳炎など）が発熱に先行する場合があり注意を要する．
- 例外的には，発熱が症状の前景に立たないで，原因の同定できない性格変化，認知障害，痴呆症状などが中核症状であれば，スローウイルス感染症，HIV 脳症，プリオン病などを考える必要がある．

参考 本邦における神経系感染症関係のデータベース

神経系感染症関係の発症動向の週報や過去の統計などが国立感染症研究所・感染症情報センター http://idsc.nih.go.jp/index-j.html のデータベースより入手可能である．また，昨今は交通手段のめざましい発達により神経系感染症の領域でも新興感染症に注意を払うべき時代になっている．ぜひこのデータベースを活用されたい．

Ⅱ どのように診断を進めていくか？

- 図 1－6－1 に神経系感染症の病原診断のためのフローチャートを示す．全身性疾患の把握と免疫不全状態やワクチン接種の有無などを含めた正確な病歴聴取と局在診断のために正しく神経学的所見をとることが肝要である．
- 同時に皮疹，水疱などの皮膚症状，耳鼻咽喉科的な異常の有無，上気道炎様症状，呼吸器症状，消化器症状などの全身状態の把握も病原診断のための一助になる．
- 季節も重要である．一般的にウイルス性髄膜炎の中で最も頻度が高いエンテロウイルスによる髄膜炎の流行季節は初夏から初秋にかけてであり，日本脳炎は夏から初秋にかけてである．
- 髄膜炎や脳炎の場合，必ずしも髄膜刺激症状（項部硬直，ケルニッヒ徴候など）が目立たない場合もある．例えば，結核性髄膜炎や真菌性髄膜炎は脳底部髄膜炎の形をとるので，しばしば髄膜刺激症状がはっきりしない場合があるので注意を要する．
- 一般的に，髄膜炎では髄膜刺激症状のみで脳の局所徴候は伴わないこと

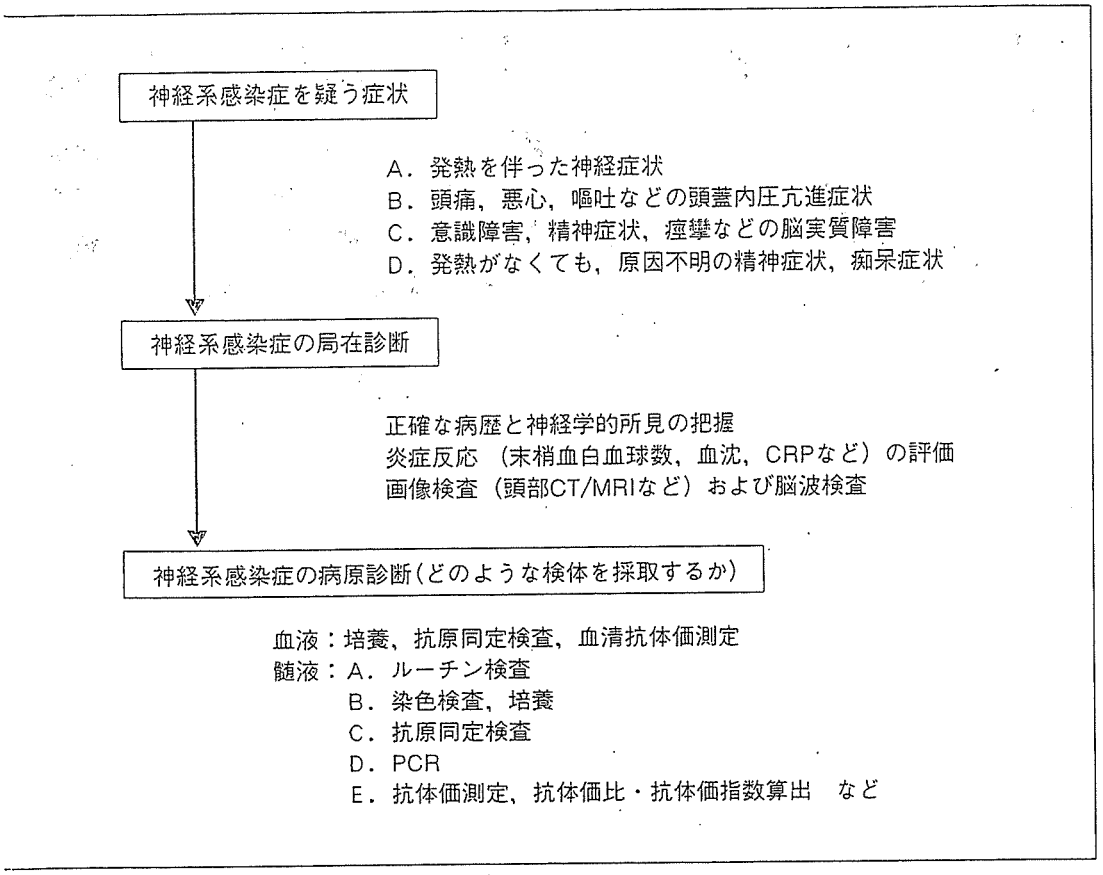

図1－6－1　神経系感染症の診断から病原体検索までのフローチャート

が多いが，動眼神経麻痺などの脳神経麻痺や片麻痺などの局所徴候が認められる場合には，結核性髄膜炎，真菌性髄膜炎，あるいは脳膿瘍などの存在を考える必要がある．

中枢神経系感染症では特異的な病型として，亜急性硬化性全脳炎（SSPE）や進行性多巣性白質脳症（PML）などのスローウイルス感染症の一群，HIV 脳症，プリオン病があり，原因がよくわからない精神症状，知能低下，認知機能の低下などがある場合は，これらの疾患も考える必要がある．

プリオン病や SSPE では不随意運動の一つであるミオクローヌスがしばしば出現する．

感染を示唆する炎症所見（末梢血白血球数，CRP など）はウイルス性髄膜炎・脳炎では異常を示さないことが多い．

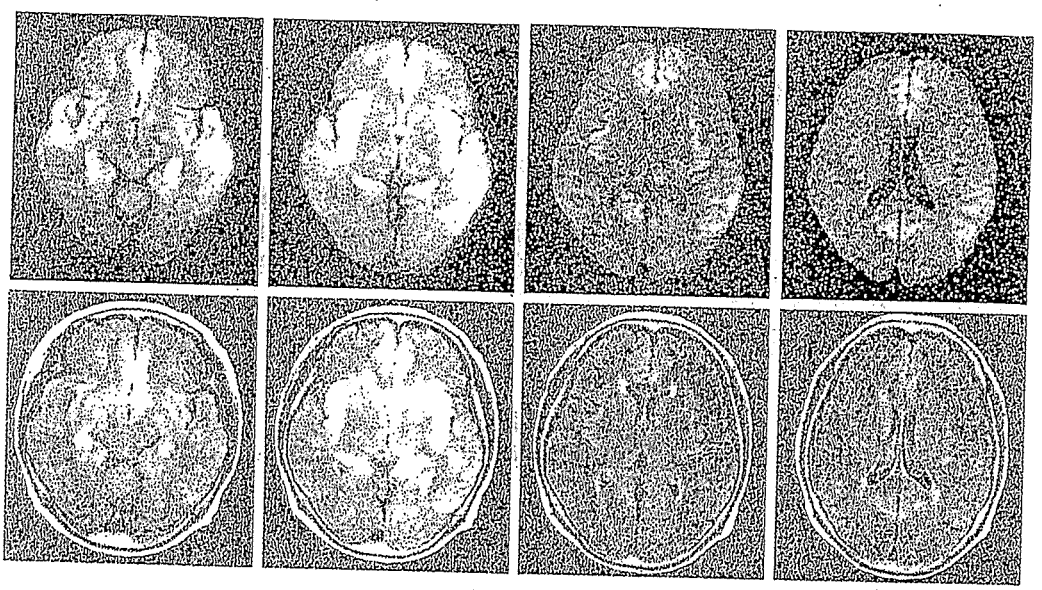

図1－6－2 a
単純ヘルペス脳炎患者（64歳，女性）の頭部MRI拡散強調画像（上段）とFLAIR画像（下段）．帯状回，島，側頭葉皮質および側頭葉内側で高信号を呈している．

- 腰椎穿刺による髄液検査は髄膜炎・脳炎の起因病原体の確定のための第一歩である．ただし，腰椎穿刺前に頭部CT検査などの画像検査を施行した方が望ましい．水頭症や脳膿瘍の合併などが疑われる場合には，腰椎穿刺は脳ヘルニアの危険性を考えて慎重であるべきである．
- 髄膜炎・脳炎の頭部CTおよびMRI検査においては必ずしも異常所見が見出されるとは限らないが，脳浮腫のための脳室の狭小化，脳溝の不鮮明化，脳槽の閉塞などがみられることがある．また，合併症として水頭症が生じれば脳室の拡大がみられたり，血管炎による脳梗塞の所見がみられることがある．
- 結核性髄膜炎の場合，脳底槽で造影剤による増強効果がみられたり，結核腫が認められることがある．
- 単純ヘルペス脳炎では前頭葉・側頭葉においてCT上低吸収域の所見やMRI上拡散強調画像，FLAIR画像，T2強調画像において異常信号域を認めることがある（図1－6－2 a）．また，日本脳炎では，大脳基底核部にMRI上T2強調画像において異常信号を認める場合がある．
- プリオン病の代表疾患である孤発性クロイツフェルトーヤコブ病（CJD）では頭部MRI拡散強調画像において皮質病変を発病の早期に検出できることがあり早期診断に有用である（図1－6－3 a）．また，一部ではT2

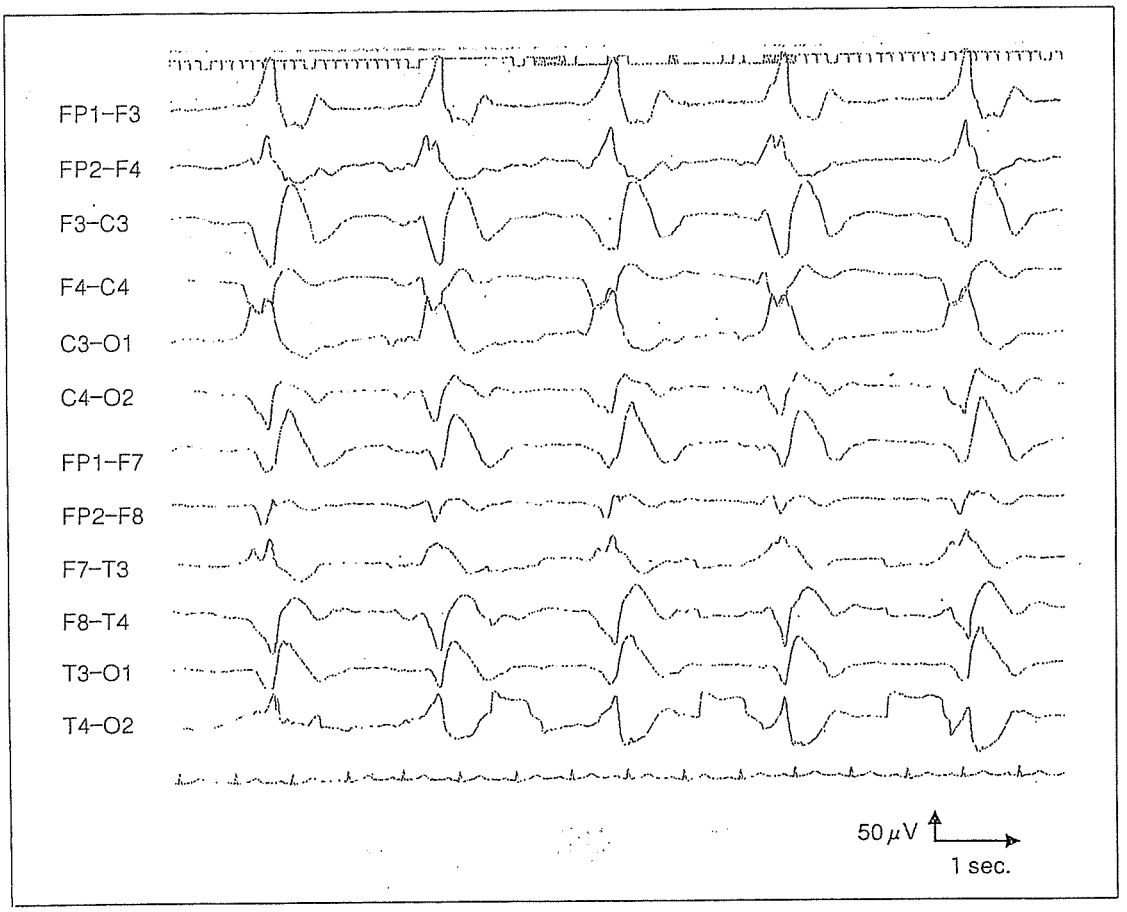

図1-6-2 b

同患者脳波検査でみられた周期性同期発射 periodic synchronous discharge (PSD). 全誘導同期性に周期的・連続性に出現している.

強調画像において発病の比較的早期より大脳基底核，視床に高信号域がみられることがある．また，SPECT で発病の早期より大脳の血流低下が検出される（図1-6-3 b）.

脳波検査は診断的特異性はあまりないが，ウイルス性脳炎の場合は汎発性徐波異常を呈することが多い.

単純ヘルペス脳炎ではほとんど全例で異常がみられ，前頭部・側頭部を中心に局所性徐波異常あるいは焦点性スパイクを呈したりする．また，周期性同期発射 periodic synchronous discharge (PSD)（図1-6-2 b）や周期性発射が一側性にみられる周期性一側てんかん形発射 periodic lateralized epileptiform discharges (PLEDs) が出現すれ

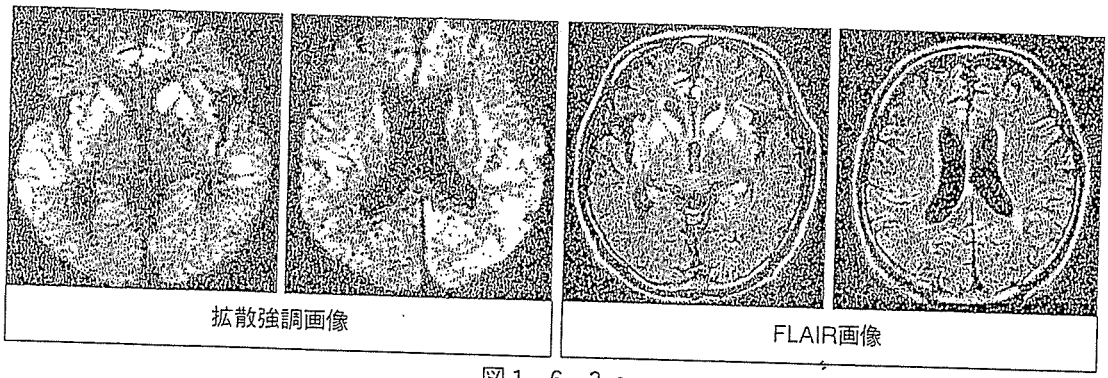

図1-6-3 a

孤発性CJD患者（67歳，男性）の発病早期の頭部MRI拡散強調画像とFLAIR画像．拡散強調画像では前頭葉，後頭葉を中心に皮質領域に高信号がみられる．また，大脳基底核領域にも高信号がみられる．

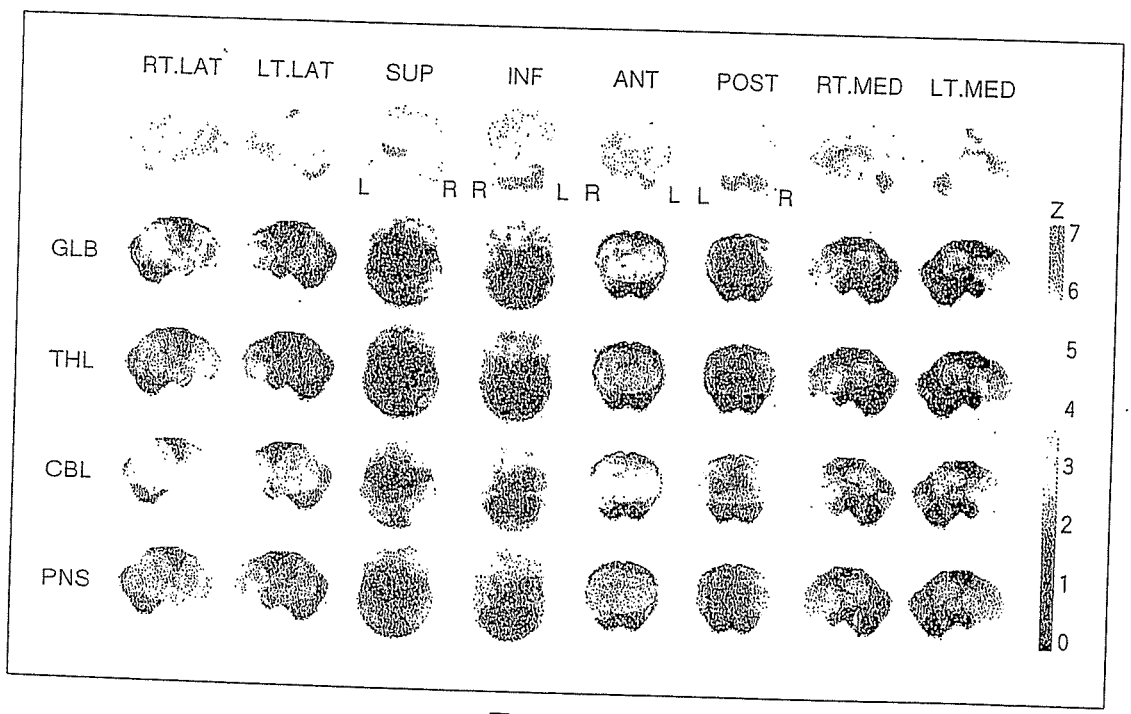

図1-6-3 b

同患者の ^{123}I-IMP SPECT所見．全般的な脳血流低下がみられる

ば診断するにあたっての意義は大きい．PSDの所見はCJDにおいてもミオクローヌスが出現する頃になるとみられることがある．