表４．平成１７年度陽性サンプルの抗HIV作用機作

検体番号	巨細胞形成抑制(%)	CCR5への作用(2D7)	逆転写酵素阻害（IC_{50}）	細胞変性抑制試験	
				MAGIC-5 (IC_{50}) μg/ml	MT-4 (IC_{100}) μg/ml
5023	0	43.7	-	5.5	-
5028	0	55.8	-	-	-
5095	0	17.7	-	4.4	-
5103	0	26.2	13.5	7.9	-
5104	0	40.8	-	14.5	10.0
5109	0	-10.9	16.9	1.7	62.5
5207	0	19.6	-	5.0	-
5505	0	63.7	8.5	15.5	0.8
陽性対照	100	70.9			

別紙　5

研究成果の刊行に関する一覧表

課題番号	H16-創薬-008
氏名	棚元憲一

著者（発表者）氏名　論文タイトル名　発表誌名　巻号，ページ，出版年

1. Shimizu, Y., Okoba, M., Yamazaki, N., Goto, Y., Miura, T., Hayami, M., Hoshino, H. and Haga T. Construction and in vitro characterization of a chimeric simian and human immunodeficiency virus with the RANTES gene. Microbes Infect. 2006; 8(1):105-13.
2. Roy BB, Jinno-Oue A, Shinagawa M, Shimizu A, Tamura K, Shimizu N, Tanaka A, and Hoshino H. Isolation of the feline alpha1,3-galactosyltransferase gene, expression in transfected human cells and its phylogenetic analysis. J Exp Zoolog B Mol Dev Evol. 2006, 306: 59-69.
3. K Abe , A Nozaki, K Tamura, M Ikeda, K Naka, H Dansako, H Hoshino, K Tanaka, and N Kato. Tandem repeats of lactoferrin-derived anti-hepatitis C virus peptide enhance antiviral activity in cultured human hepatocytes. Microbiol Immunol. 2007, 51: 117-125.
4. Phan TG, Kuroiwa T, Kaneshi K, Ueda Y, Nakaya S, Nishimura S, Yamamoto A, Sugita K, Nishimura T, Yagyu F, Okitsu S, Müller WEG, Maneekarn N, Ushijima H. Changing Distribution of Norovirus Genotypes and Genetic Characterization of Recombinant GIIb among Infants and Children with Diarrhea in Japan. J Med Virol. 78(7): 971-978, 2006.
5. Phan TG, Yagyu F, Kozlov V, Kozlov A, Okitsu S, Müller WEG, Ushijima H. Viral gastroenteritis and Genetic Characterization of Recombinant Norovirus among Infants and Children with Diarrhea in Eastern Russia. Clin Lab 52 (5-6): 247-253, 2006.
6. Phan TG, Yan H, Li Y, Okitsu S, Müller WEG, Ushijima H. Novel Recombinant Norovirus in China. Emerg Infect Dis. 12(5): 857-858, 2006.
7. Phan TG, Okitsu S, Müller WEG, Kohno H, Ushijima H. Identification of Novel Recombinant Sapovirus in Japan. Emerg Infect Dis. 12(5): 865-867, 2006.
8. Khamrin P, Maneekern N, Peerakome S, Yagyu F, Okitsu S, Ushijima H. Molecular characterization of a rare G3P[3] human rotavirus reassortant strain reveals an evidence for human-animals multiple interspecies transmissions. J Med Virol 78(7):986-994, 2006.
9. Phan TG, Trinh OD, Yagyu F, Sugita K, Okitsu S, Muller WEG, Ushijima H. Outbreak of sapovirus infection among infants and children with acute gastroenteritis in Osaka City, Japan during during 2004-2005. J Med Virol 78(6):839-846, 2006.
10. Okame M, Akihara S, Hansman G, hainan Y, Thien Tuan Tran H, Phan TG, Yagyu F, Okitsu S, Ushijima H. Existence of multiple genotypes associated with acute gastroenteritis during 6-year survey of norovirus infection in Japan. J Med Virol 78(10):1318-1324, 2006.
11. Phan TG, Shimizu H, Okitsu S, Maneekarn N, Ushijima H. Human adenovirus type 1 related to feline adenovirus: evidence of interspecies transmission. Clin Lab 52 (9-10): 515-518, 2006.
12. Phan TG, Takanashi S, Kaneshi K, Ueda Y, Nakaya S, Nishimura S, Sugita K, Nishimura T,Yamamoto A, Yagyu F, Okitsu S, Ushijima H. Detection and genetic characterization of norovirus strains circulating among infants and children with acute gastroenteritis in Japan during 2004-2005. Clin Lab 52 (9-10): 519-525, 2006.
13. Phan TG, Yan H, Khamrin P, Quang T, Dey SK, Yagyu F, Okitsu S, Mueller WEG, Ushijima H. Novel intragenotype recombination in sapovirus. Clin Lab 52(7-8):363-366, 2006.
14. Okitsu-Negishi S, Okame M, Shimizu Y, Phan TG, Tomaru T, Kamijo S, Sato T, Yagyu F, Mueller WEG, Ushijima H. Detection of norovirus antigens from recombinant virus-like particles and stool samples by a commercial norovirus enzyme-linked immunosorbent assay. J Clin Microbiol 44(10):3784-3786, 2006.
15. Phan TG, Khamrin P, Quang TD, Dey SK, Yagyu F, Okitsu S, Nishio O, Ushijima H. Genetic characterization of group A rotavirus strains circulating among children with acute gastroenteritis in Japan in 2004-2005. Infection, Genetics and Evolution 7: 247-253, 2007.
16. Phan TG, Trinh QD, Kaneshi K, Ueda Y, Nakaya S, Nishimura S, Sugita K, Nishimura T, Yamamoto A, Yagyu F, Okitsu S, Ushijima H. Emergence of new variant rotavirus G3 among infants and children with acute gastroenteritis in Japan during 2003-2004. Clin Lab, 53: 41-48, 2007.
17. Maneekarn N, Khamrin P, Chan-it W, Peerakome S, Sukchai S, Pringprao K, Ushijima H. Detection of rare G3P[19] porcine rotavirus strains in Chiang Mai, Thailand provides evidence for the origin of VP4 genes of Mc323 and Mc345 human rotaviruses. J Clin Microbiol 44:4113-4119, 2006.
18. Shimizu H, Phan TG, Nishimura S, Okitsu S, Maneekarn N, Ushijima H. An outbreak of adenovirus serotype 41 infection in infants and children with acute gastroenteritis in Maizuru city, Japan. Infect, Genet and Evol . 7:279-284,

2007

19. Phan TG, Trinh QD, Yagyu F, Okitsu S, Ushijima H. Emergence of rare sapovirus genotype among infants and children with acute gastroenteritis in Japan. European J Clin Microbiol & Infect Diseases 26(1): 21-27,2007.
20. Zhou Y, Ushijima H, Frey TK. Genomic analyses of diverse rubell virus genotypes. J Gen Virol 88:932-941, 2007.
21. Khamrin P, Maneekarn N, Peerakome S, Chan-It W, Yagyu F, Okitsu S, Ushijima H. Norvel porcine rotavirus of the genotype P[27] shares new phylogenetic lineage with G2 porcine rotavirus strain. Virology 2007 (E-pub).
22. Sasaki Y, Kai A, Hayashi Y, Shinkai T, Noguchi Y, Hasegawa M, Sadamasu K, Mori K, Tabei Y, Nagashima M, Morozumi S, Yamamoto T. Multiple Viral Infections and Genomic Divergence among Noroviruses during an Outbreak of Acute Gastroenteritis. J Clin Microbiol. 44,790-797,2006
23. Muroi M. and Tanamoto K. Structural regions of MD-2 that determine the agonist-antagonist activity of lipid IVa. J. Biol. Chem. 281, 5484-5491, 2006
24. Igarashi A., Ohtsu S., Muroi M. and Tanamoto K. Effects of possible endocrine disrupting chemicals on bacterial component-induced activation of NF-κB. Biol. Pharm. Bull. 29, 2120-2122, 2006
25. Raphael Lwembe, Washingtone Ochieng, Annie Panikulam, Charles O. Mongoina, Tresa Palakudy, Mary Owens, Yusuke Koizumi, Seiji Kageyama, Naohiko Yamamoto, Tatsuo Shioda, Rachel Musoke, Angelo D'Agostino, Elijah M. Songok, Frederick A. Okoth, and Hiroshi Ichimura. Anti-retroviral drug resistance-associated mutations among non-subtype B HIV-1-infected Kenyan children with treatment failure. the Journal of Medical Virology. (in press)
26. 川畑拓也、小島洋子、森　治代、大竹　徹、大國　剛、当所にて HIV 感染を確認した,2 例のイムノクロマトグラフィー法陰性の感染初期例、感染症学雑誌　81, 76-77, 2007
27. Huy TT, Ishikawa K, Ampofo W, Izumi T, Nakajima A, Ansah J, Tetteh JO, Nii-Trebi N, Aidoo S, Ofori-Adjei D, Sata T, Ushijima H, Abe K. Characteristics of hepatitis B virus in Ghana: full length genome sequences indicate the endemicity of genotype E in West Africa. J Med Virol. 2006 Feb;78(2):178-84.
28. Khamrin P, Peerakome S, Wongsawasdi L, Tonusin S, Sornchai P, Maneerat V, Khamwan C, Yagyu F, Okitsu S, Ushijima H, Maneekarn N. Emergence of human G9 rotavirus with an exceptionally high frequency in children admitted to hospital with diarrhea in Chiang Mai, Thailand. J Med Virol. 2006 Feb;78(2):273-80.
29. Huy TT, Ushijima H, Sata T, Abe K. Genomic characterization of HBV genotype F in Bolivia: genotype F subgenotypes correlate with geographic distribution and T(1858) variant. Arch Virol. 2006 Mar;151(3):589-97.
30. Akihara S, Phan TG, Nguyen TA, Yagyu F, Okitsu S, Muller WE, Ushijima H. Identification of sapovirus infection among Japanese infants in a day care center. J Med Virol. 2005 Dec;77(4):595-601.
31. Yagyu F, Okitsu S, Tanamoto K, Ushijima H. Determination of HIV-1 subtypes (A-D, F, G, CRF01_AE) by PCR in the transmembrane region (gp41) with novel primers. J Med Virol. 2005 May;76(1):16-23.
32. Phan TG, Nguyen TA, Shimizu H, Yagyu F, Okitsu S, Muller WE, Ushijima H. Related Identification of enteroviral infection among infants and children admitted to hospital with acute gastroentritis in Ho Chi Minh City, Vietnam. J Med Virol. 2005 Oct;77(2):257-64.
33. Yoshinaga M, Phan TG, Nguyen TA, Yan H, Yagyu F, Okitsu S, Muller WE, Ushijima H. Changing distribution of group A rotavirus G-types and genetic analysis of G9 circulating in Japan. Arch Virol. 2006 Jan:183-192
34. Akihara S, Phan TG, Nguyen TA, Hansman G, Okitsu S, Ushijima H. Existence of multiple outbreaks of viral gastroenteritis among infants in a day care center in Japan. Arch Virol. 2005 Oct;150(10):2061-75.
35. Phan TG, Nguyen TA, Kuroiwa T, Kaneshi K, Ueda Y, Nakaya S, Nishimura S, Nishimura T, Yamamoto A, Okitsu S, Ushijima H. Viral diarrhea in Japanese children: results from a one-year epidemiologic study. Clin Lab. 2005;51(3-4):183-91.
36. Phan TG, Nguyen TA, Nishimura S, Nishimura T, Yamamoto A, Okitsu S, Ushijima H. Etiologic agents of acute gastroenteritis among Japanese infants and children: virus diversity and genetic analysis of sapovirus. Arch Virol. 2005 Jul;150(7):1415-24.
37. Li L, Phan TG, Nguyen TA, Kim KS, Seo JK, Shimizu H, Suzuki E, Okitsu S, Ushijima H. Molecular epidemiology of adenovirus infection among pediatric population with diarrhea in Asia. Microbiol Immunol. 2005;49(2):121-8.
38. Yan H, Abe T, Phan TG, Nguyen TA, Iso T, Ikezawa Y, Ishii K, Okitsu S, Ushijima H. Outbreak of acute gastroenteritis associated with group A rotavirus and genogroup I sapovirus among adults in a mental health care facility in Japan. J Med Virol. 2005 Mar;75(3):475-81.
39. Phan TG, Nguyen TA, Yan H, Okitsu S, Ushijima H. A novel RT-multiplex PCR for enteroviruses, hepatitis A and E viruses and influenza A virus among infants and children with diarrhea in Vietnam. Arch Virol. 2005 Jun;150(6):1175-85.
40. Phan TG, Okame M, Nguyen TA, Nishio O, Okitsu S, Ushijima H. Genetic diversity of sapovirus in fecal specimens from infants and children with acute gastroenteritis in Pakistan. Arch Virol. 2005 Feb;150(2):371-7.
41. 長島真美、貞升健志、新開敬行、秋場哲哉、吉田　勲、吉田靖子、矢野一好、甲斐明美、諸角　聖、東京都における HIV 検査成績(1999 年-2004 年)、東京都健康安全研究センター年報、56,2005（印刷中）
42. Sasaki Y, Kai A, Hayashi Y, Shinkai T, Noguchi Y, Hasegawa M, Sadamasu K, Mori K, Tabei Y, Nagashima M, Morozumi S, Yamamoto T. Multiple Viral Infections and Genomic Divergence among Noroviruses during an Outbreak of Acute Gastroenteritis.
43. J Clin Microbiol. 44,790-797,2006
44. Otake T, Kawahata T, Mori H, Kojima Y, Hayakawa K, Novel method of inactivation of human immunodeficiency

virus type 1 by the freeze pressure generation method, Applied Microbiology and Biotechnology, 67, 746-751, 2005

45. New estimation method for highly sensitive quantitatiion of human immunodeficiency virus type 1 DNA and its application; H. Nagai, K. Wada, T. Morishita, M. Utsumi, Y. Nishiyama and T. Kaneda J. Virol. Methods 124, 157-165 (2005)
46. Roy BB, Jinno-Oue A, Shinagawa M, Shimizu A, Tamura K, Shimizu N, Tanaka A, and Hoshino H. Isolation of the feline alpha1,3-galactosyltransferase gene, expression in transfected human cells and its phylogenetic analysis. J. Exp.
47. Saha, M. N., Tanaka, A., Jinno-Oue, A., Shimizu, N., Tamura, K., Shinagawa, M., Chiba, J., and Hoshino H. Formation of vesicular stomatitis virus pseudotypes bearing surface proteins of hepatitis B virus. J. Virol. 2005, 79, 12566-12574.
48. Jinno-Oue A, Shimizu N, Soda Y, Tanaka A, Ohtsuki T, Kurosaki D, Suzuki Y, and Hoshino H. The synthetic peptide derived from the NH2-terminal extracellular region of an orphan G protein-coupled receptor, GPR1, preferentially inhibits infection of X4 human immunodeficiency virus type 1. J. Biol. Chem. 2005, 280, 30924-30934.
49. Tamura K, Oue A, Tanaka A, Shimizu N, Takagi H, Kato N, Morikawa A, and Hoshino H. Efficient formation of vesicular stomatitis virus pseudotypes bearing the native forms of hepatitis C virus envelope proteins detected after sonication. Microbes Infect. 2005, 7: 29-40
50. Yagyu F, et al.Determination of HIV-1 subtypes (A, B, C, D, G, CRF01_AE) by PCR in the transmembrane region (gp41) with novel primers. J Med Virol In press
51. Phan TG, et al. Etiologic agents of acute gastroenteritis among Japanese infants and children: virus diversity and genetic analysis of sapovirus. Arch Virol In press
52. Hansman GS, et al. Characterization of polyclonal antibodies raised against five virus-like particles. Arch Virol In press
53. Oka T, et al. Proteolytic processing of sapovirus ORF1 polyprotein. J Virol In press
54. Phan TG, et al.A novel RT-multiplex PCR for enteroviruses, hepatitis A and E viruses and influenza A virus among infants and children with diarrhea in Vietnam. Arch Virol In press
55. Li L, et al. Molecular Epidemiology of Adenovirus Infection among Pediatric Population with Diarrhea in Asia. Microbiol Immunol 49(2):121-128, 2005
56. Phan TG, et al.Genetic diversity of sapovirus in fecal specimens from infants and children with acute gastroenteritis in Pakistan. Arch Virol 150(2):371-377, 2005
57. Yan H, et al.Outbreak of acute gastroenteritis associated with group A rotavirus and genogroup I sapovirus among adults in a mental health care facility in Japan. J Med Virol 75(3):475-481, 2005
58. Hansman GS, et al. Viral gastroenteritis in Mongolian infants. Emerg Infect Dis 11(1): 180-182, 2005
59. Hansman GS, et al. Cross-reactivity among sapovirus recombinant capsid proteins. Arch Virol 150(1):21-36, 2005
60. Li L, et al. Characterizations of adenovirus type 41 isolates from children with acute gastroenteritis in Japan, Vietnam, and Korea. J Clin Microbiol 42(9):4032-4039, 2004
61. Phan TG, et al.Virus diversity and an outbreak of group C rotavirus among infants and children with diarrhea in Maizuru city, Japan during 2002-2003. J Med Virol 74(1):173-179, 2004
62. Yan H, et al. Development of RT-multiplex PCR assay for detection of adenovirus and group A and C rotaviruses in diarrheal fecal specimens for children in China. Kansenshogakuzasshi 78(8):699-709, 2004
63. Hansman GS, et al. Detection of norovirus and sapovirus infection among children with gastroenteritis in Ho Chi Minh City, Vietnam. Arch Virol 149(9):1673-1688, 2004
64. Phan TG, et al.Human astrovirus, norovirus (GI, GII), and sapovirus infections in Pakistani children with diarrhea. J Med Virol 73(2):256-261, 2004
65. Okitsu-Negishi S, et al.Molecular epidemiology of viral gastroenteritis in Asia. Pediatr Int 46(2):245-252, 2004
66. Ushijima H and Eshita Y. Foreward: Molecular epidemiology of viral infection in Asia. Pediatr Int 46(2):202-206, 2004
67. Sakamoto T, et al. Establishment of an HIV cell-cell fusion assay by using two genetically modified HeLa cell lines and receptor gene. J Virol Methods 114:159-166, 2004
68. Hansman GS Genetic diversity of Norovirus and Sapovirus in hospitalized infants with sporadeic cases of acute gastroenteritis in Chiang Mai, Thailand. J Clin Microbiol 42:1305-1307, 2004
69. Adhikary AK, et al. Characterization of hexon and fiber genes of a novel strain of adenovirus involved in epidemic keratoconjunctivitis. J Clin Pathol 57:95-97, 2004
70. Tran TTH, et al.Genotypic C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 85:283-292, 2004
71. Tran TTH, et al. Characteristics of core promoter and precore stop codon mutants of hepatitis B virus in Vietnam. J Med Virol 74:228-236, 2004
72. Urata H, Kumashiro T, Kawahata T, Otake T, Akagi M, Anti-HIV-1 activity and mode of action of mirror image oligodeoxynucleotide analogue of zintevir, Biochemical and Biophysical Research Communications, 313, 55-61, 2004
73. 森 治代、小島洋子、川畑拓也、大竹 徹、巽 正志、コレセプター阻害剤を用いた R5/X4 ウイルス測定法、MINOPHAGEN MEDICAL REVIEW vol.49, 81-82, 2004

74. Dan Turner, Bluma Brenner, Daniela Moisi, Mervi Detorio, Raymond Cesaire, Takashi Kurimura, Haruyo Mori, Max Essex, Shlomo Maayan and Mark A. Wainberg, Nucleotide and Amino Acid Polymorphisms at Drug Resistance Sites in Non-B-Subtype Variants of Human Immunodeficiency Virus Type 1, Antimicrobial Agents and Chemotherapy, 48, 2993-2998, 2004
75. 大竹　徹、ウイルスの高圧不活化と血液製剤への利用、FFI ジャーナル、210(1)、44-48、2005
76. Tamura K, Oue A, Tanaka A, Shimizu N, Takagi H, Kato N, Morikawa A, and Hoshino H. Efficient formation of vesicular stomatitis virus pseudotypes bearing the native forms of hepatitis C virus envelope proteins detected after sonication. Microbes Infect. 2005, 7: 29-40.
77. Shimizu A, Shimizu N, Tanaka A, Jinno-Oue A, Roy BB, Shinagawa M, Ishikawa O, and Hoshino H. Human T-cell leukaemia virus type I is highly sensitive to UV-C light. J. Gen. Virol. 2004. 85:2397-406.

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抗エイズ新薬開発に関する研究

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研究成果の刊行物・別刷

Correspondence

Emerging role of the private sector in HIV/AIDS disease prevention in Pakistan

HIV/AIDS in Pakistan is slowly gaining recognition as a public health issue of great importance. As in many developing countries, the HIV/AIDS epidemic continues to spread in Pakistan, despite the efforts of many committed partners throughout the country. The country's still relatively low HIV prevalence, however, presents an enormous opportunity to prevent the unleashing of a major epidemic.

A decade has passed since the discovery of HIV in Pakistan. According to the UNAIDS, Pakistan has a high-risk/low-prevalence status for HIV/AIDS but this could quickly change to a high-risk/high-prevalence status. Pakistan's National AIDS Programme claims a prevalence of 0.07% and has reported 216 AIDS and 1639 HIV-positive cases[1]. Pakistan is currently spending less than US$0.01 per person per year on its control and prevention and less than 10% of the blood transfused in the country is tested for HIV[2] In the past few years political commitment[3] and financial support for HIV/ AIDS projects have been declining[2,4], and this coupled with the extremely low awareness of HIV/AIDS in Pakistan, means that the AIDS epidemic is likely to take a hold in Pakistan.

Faced with declining public resources, governments have been forced to accept the private sector as a necessary participant in pursuing their health care agendas. Collaboration between the public sector and non-governmental organizations (NGOs), in the form of public–private partnerships to pursue health goals, offers enormous potential for mustering vast human, financial and technical resources. NGOs can predominantly contribute by creating awareness at the grass root level, as they have the best access to most vulnerable population groups.

Currently, at least 72 local NGOs[5] are involved in HIV/ AIDS-related activities throughout the country. During January and February 2001, 58 NGOs[5] working for HIV/ AIDS prevention, underwent a cross sectional survey, using a semi-structured questionnaire. The aim of the survey was to collate information on NGOs working for HIV/AIDS prevention in Pakistan, with a view to bringing together their variety of experiences for mutual benefit. This study explored the role of NGOs by identifying their activities, target groups and educational methodology for disease prevention among the high-risk groups. Here we report on the interviews conducted with the NGOs.

The results indicated that NGOs had a wide range of activities including: awareness-raising through mass media; HIV/AIDS prevention materials production; prevention activities among high-risk groups; peer education programmes; safe blood programmes; condom promotion; training of health professionals and educators; sensitization of political and other influential leaders; and clinical and behavioural research. Most NGOs were concentrated in two provinces (Punjab and Sindh) thus pointing to the need for expanding activities in other areas having vulnerable populations.

Although most of the NGOs seem to use the curricula developed by the National AIDS Programme and UNICEF in awareness campaigns few NGOs used their own course, indicating the need to standardize educational material. The NGOs used workshops/seminars, peer education and interpersonal communication in educating their target groups They focused on disease definition, signs and symptoms, mode of transmission, and benefits of condom use. Provision of practical skills in proper condom use should be emphasized.

Most of the NGOs were working on high-risk target groups ranging from youth, adult men and women to commercial sex workers, truck drivers, prisoners, AIDS patients, health care providers and injecting drug users. The results highlight the need for increased focus on these high-risk groups (see Figure 1).

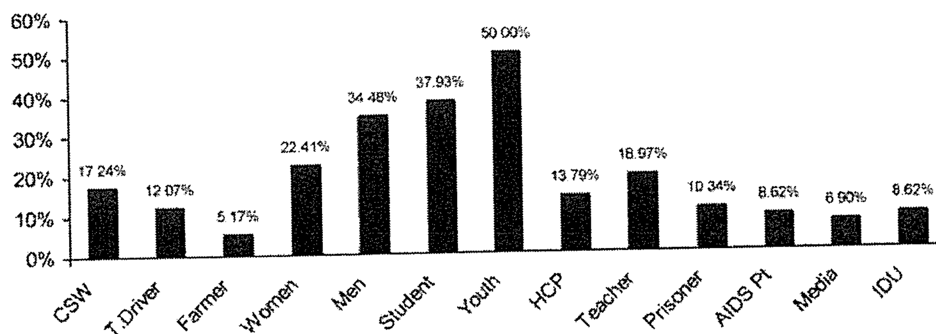

Fig.1 Target groups for HIV/AIDS education (n=58) HCP=health care provider; CSW=commercial sex worker; IDU=injecting drug users; T-Driver=truck driver; youth=out of school male youth

Our findings suggest that NGOs ought to be encouraged to engage in research and information sharing activities with other partner NGOs. They should exchange their experiences on best practices in teaching methodology, similar target groups and condom provision to the difficult-to-reach groups.

Pakistan is at a crucial juncture in its response to the threat of HIV/AIDS. It has a narrow window of opportunity to act immediately and decisively to prevent a widespread HIV/AIDS epidemic. A multi-sectoral approach by efficient networking between the public and private sectors and timely steps may help to prevent the onslaught of an HIV/AIDS epidemic in Pakistan.

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References

1 Pakistan National AIDS Control Programme Report. September 2001. Islamabad: Ministry of Health
2 Khabir Ahmad. Pakistan investigates black market sale of HIV kits. Lancet 2001; 357(9264):1269
3 Hyder AA, Khan OA, Shah SA, Memon MA, Khanani MR, Ali S. Sub-national response in HIV/AIDS: a case study in been AIDS prevention and control from Sindh province, Pakistan. Public Health 1999;113(1):39–43
4 Ministry of Health and UNAIDS. HIV/AIDS in Pakistan. A situation and response analysis. 2000. UNAIDS. NGOs working in HIV/AIDS prevention in Pakistan, 2000

Journal of Medical Virology 76:16–23 (2005)

Determination of HIV-1 Subtypes (A–D, F, G, CRF01_AE) by PCR in the Transmembrane Region (gp41) With Novel Primers

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HIV-1 has a huge genetic diversity. So far, nine subtypes have been isolated, namely, subtypes A, B, C, D, F, G, H, J, and K. Epidemiological study provides information which may help in the development of HIV-1 prevention programs or health policies. In the future, subtyping may also be critical for vaccine development, and an effective anti-viral drug will need to be effective for different subtypes of HIV virus. The analysis of the nucleotide sequence of the v3 region is considered the most reliable method for determining the HIV-1 subtype. However, the procedures for determining the v3 sequences are complicated and time consuming, requiring expensive reagents, equipment, and well-trained personnel. The polymerase chain reaction (PCR) method using subtype-specific primers for HIV-1 subtyping is easier and faster. The objective of this study was to develop subtype-specific primers for subtyping PCR. The specific primers were designed for subtypes A, B, C, D, F, G, and CRF01_AE, and these primers could be applied to assay for various HIV-1 subtypes in the clinical samples. The specific primers were designed for each subtypes in the gp41 region. The result of PCR was compared with the subtypes which was determined by the v3 sequence. The results of subtyping by PCR using the newly designed primers could detect 29 of 33 patients tested, and all matched those obtained by nucleotide sequencing of the env v3 region except for three subjects, which were differentiated as CRF02_AG. The newly designed primers functioned accurately and conclusively. In comparison with PCR as a method for the determination of subtypes, sequence analysis requires better-trained personnel, more expensive reagents, and more equipment and time. ***J. Med. Virol. 76:16–23, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HIV-1; subtyping; PCR

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INTRODUCTION

HIV-1 has numerous genetic variations and can be divided by group, subtype, and subsubtype. The majority of HIV-1 strains belong to the M (Major) group, which causes pandemic HIV-1 infection. The O (Outlier) group and the N (Non-M/Non-O) group are seldomly found. Both the O and N groups have high genetic diversity from the M group [Charneau et al., 1994; Gurtler et al., 1994; Vanden Haesevelde et al., 1994; Loussert-Ajaka et al., 1995; Simon et al., 1998].

The M group has thus far been subdivided into nine isolated subtypes: A, B, C, D, F, G, H, J, and K [Carr et al., 1998; Robertson et al., 2000]. Although some isolates from Cyprus and Greece (94CY032, PVMY, and PVCH) were recognized previously as subtype I, they were shown to be a recombinant strain upon reanalysis. Subtype K was recognized initially as subtype F3, and later designated subtype K. Subtypes A and F are divided, respectively, into subsubtypes A1 and A2, and F1 and F2 [Triques et al., 1999, 2000]. Subtypes B and D are similar [Robertson et al., 2000; Triques et al., 2000], and although they should be reclassified as a single subtype, for the sake of historical consistency, they remain classified as different subtypes.

HIV-1 has been divided into not only many subtypes but also many circulating recombinant forms (CRFs), and so far 15 CRFs have been isolated [Carr et al., 1998]. For example, in the CRF01_AE strain, only the env region belongs to subtype E; the rest of the regions

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belong to subtype A, and the original full-length of subtype E is no longer found [Carr et al., 1996; Gao et al., 1996].

Worldwide, the incidence of HIV-1 subtypes is as follows: subtype C (47.0%), subtype A (27.2%), subtype B (12.3%), subtype D (5.3%), and CRF01_AE (3.2%) [Osmanov et al., 2002]. Certain subtypes prevail in distinct geographical areas, and different subtypes may predominate for each of the different means of infection [Essex, 1999]. In Thailand, subtype B is predominant among those infected through intravenous drug use, and subtype E among those infected through sexual intercourse [Ou et al., 1992, 1993; Weniger and Brown, 1996; Lole et al., 1999].

The gp120 of the HIV-1 envelope gene comprises five variable domains interspersed with conserved regions. The third variable (v3) region plays an important role in biological properties such as cell tropism, cytopathic effect, and pathogenicity [Robert-Guroff et al., 1994; Palker et al., 1988; Takahashi et al., 1988]. Therefore, analysis of the nucleotide sequence of the v3 region is considered as the most reliable method for determining the HIV-1 subtype. However, the procedures for determining the v3 sequences are complicated and time consuming, requiring expensive reagents, equipment, and well-trained personnel. For determining HIV-1 subtypes of a large number of samples, the heteroduplex mobility assay (HMA) and the peptide enzyme-linked immunosorbent assays (PELISAs) are usually used [Cheingsong-Popov et al., 1994; Wasi et al., 1995; Gaywee et al., 1996; Novitsky et al., 1996; Delwart et al., 1998]. Both of these methods often show cross-reaction or are not reliable. Recently, new methods were attempted to detect subtypes and recombinants [Hoelscher et al., 2002; Plantier et al., 2002]. However, these new methods are more difficult and complicated than PCR. Furthermore, the cost of the single PCR method in particular is approximately 10 times less than that of the sequence method, and thus consideration should be given to its preferential use in developing countries in the future [Yagyu et al., 2002].

The objective of this study was to develop subtype-specific primers for subtyping PCR, since the PCR method is easy, fast, cheap, and accurate. Therefore, specific primers were designed for subtypes A, B, C, D, F, G, and CRF01_AE, and these primers could be applied to examine various HIV-1 subtypes in clinical samples. This method could be applied with several advantages in developing countries, which face a large number of people living with HIV/AIDS.

MATERIALS AND METHODS

Clinical Specimens

Blood samples were collected from HIV-1 carriers with informed consent (Table I). All the subjects were positive originally for anti-HIV-1 antibody. Six samples were from Brazil and the patients had a history of treatment. Six samples from Japan, 2 samples from Kenya, 2 samples from Thailand, 1 sample from Tanzania, and 15 samples from Africa, for all of which a history of treatment was not available. The collection site of one sample was not specified nor the history of medication was known. Blood sample was obtained without any anti-coagulant and incubated at 56°C for 1 hr and stored at −20°C.

DNA Extraction

Stored samples were thawed at room temperature, centrifuged at 12,000*g*, and the supernatants were discarded. The cell pellets were suspended in 500 µl of DNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA), 5 µl of 10% SDS and 5 µl of Proteinase K (25 mg/ml) were added, and incubated at 56°C for 1 hr. Following phenol and phenol/chloroform extractions, DNA was ethanol-precipitated at −85°C for 1 hr. After centrifugation at 12,000*g* for 15 min, DNA pellets were rinsed with ice-cold 70% ethanol and dried. The dried pellet in each tube was dissolved in 15 µl of distilled H_2O and stored at −20°C until use.

PCR of Cellular Beta-Actin

To examine the integrity of the DNA samples, cellular *beta-actin* gene was amplified as described previously with b-F and b-R primers [Yagyu et al., 2002] (Table II). The PCR products were subjected to 1% agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide.

Differentiation of Subtype B and E by PCR With Subtype-Specific Primers

Nested PCR with two sets of primers were carried out for differentiating subtypes B and E as described previously [Yagyu et al., 2002]. The primers were BECO5 and BECO3 for the first round and BE-ANCH, B-SPEC, and E-SPEC for the second round in PCR reaction (Table II). The PCR products were subjected to 1% agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide.

Determination of Subtypes by PCR With Novel Designed Primers

The primers were BECO5 and BECO3 for the first round and BE-ANCH, B-SPEC, C-SPEC, E-SPEC, and F-SPEC for the second round in PCR reaction (Table II, Fig. 1). The primers were 5′D and 3′D, or 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the alternative second round PCR (Table II).

A reaction mixture was made by adding 5.0 µl of 10 × PCR buffer with 22.5 mM $MgCl_2$ (Roche Diagnostics, Indianapolis, IN), 0.3 µl of enzyme mix (Roche Diagnostics), 1.5 µl each of dATP, dCTP, dGTP, and dTTP (25 mM each), 1.5 µl each primer (33 pM each), 1.0 µl of template DNA solution and adding distilled H_2O, up to 50 µl. The cycle condition was 93°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min, for 30 cycles.

The second round PCR products were subjected to 2% agarose gel electrophoresis at 100 V for 1 hr and stained with ethidium bromide.

TABLE I. Samples and Subtyping Results

Sample	Sex	Age	Sample taken in	HV+ from when	Symptom	The way of infection	Drug therapy	Sampling date	v3 sequence	PCR differentiate subtype B and E[b]	PCR with newly designed primer[c]	PCR with newly designed primer[d]	PCR with newly designed primer[e]
BRON01	M	34	Brazil	Sep/95	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON02	M	24	Brazil	Aug/95	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON03	M	54	Brazil	Nov/96	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON04	F	31	Brazil	Aug/98	AC	Sexual	+	Nov/99	F	—[f]	F	—[g]	—[g]
BRON05	M	33	Brazil	Mar/97	AIDS	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON06	M	36	Brazil	Mar/93	AIDS	Sexual	+	Nov/99	B	B	B	—[g]	B
YoD	—[a]	—[a]	Japan	—[a]	—[a]	—[a]	—[a]	—[a]	D	B	B	—[g]	D
OS01	F	—[a]	Tanzania	—[a]	—[a]	Sexual	—[a]	—[a]	D	B	B	—[g]	D
OS02	F	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	D	B	B	—[g]	D
OS03	F	—[a]	Thailand	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS04	F	—[a]	Thailand	—[a]	—[a]	Blood transfusion	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS05	M	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS06	—[a]	—[a]	—[a]	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS07	F	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS08	F	—[a]	Kenya	—[a]	—[a]	Sexual	—[a]	—[a]	CRF02 AG	CRF01 AE	CRF01 AE	A	—[g]
OS09	M	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS10	F	—[a]	Kenya	—[a]	—[a]	Blood transfusion	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]
NG01	F	41	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG02	F	38	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	—[f]	—[f]	—[f]	—[g]
NG03	F	21	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	B	B	—[f]	D
NG04	M	14	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG05	F	36	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG06	F	26	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	CRF02 AG	CRF01 AE	CRF01 AE	A	—[g]
NG07	F	20	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG08	F	61	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	CRF02 AG	—[f]	—[f]	—[g]	—[g]
NG09	F	34	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG10	F	35	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG11	M	33	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	B	B	—[g]	D
NG12	M	40	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	—[f]	—[f]	—[g]	—[f]
JP02	M	31	Japan	1994	AC	Sexual	+	Jul/1999	B	—[f]	—[f]	—[g]	—[f]
KA14	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]
KA18	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	F	—[f]	—[f]	—[g]	—[g]
KA43	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]

[a] No data.
[b] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, and E-SPEC for the second PCR.
[c] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers C-SPEC and F-SPEC for the second round PCR.
[d] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the second round PCR.
[e] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers 5′D and 3′D for the second round PCR.
[f] No product generated.
[g] Not done.

TABLE II. Sequence of Primers and Positions

Name	Sequence (5′–3′)	HXB2 no.[a]
J5′-2KSI	ATAAGCTTGCAGTGTAGCAGAAGAAGA	7003–7029
5′C2V3	TGTACACATGGAATTAGGCCAG	6963–6984
3′V3	ATGAATTCATTACAGTAGAAAAATTCCC	7363–7391
3′C2V3	ATTTCTGGGTCCCCTCCTGAGG	7313–7334
BECO5	GGCATCAAACAGCTCCAGGCAAG	7938–7960
BECO3	AGCAAAGCCCTTTCTAAGCCCTGTCT	8766–8791
BE-ANCH	TCCTGGCTGTGGAAAGATACCTA	7963–7985
B-SPEC	GTCCCCTCGGGGCTGGGAGG	8384–8403
E-SPEC	GTCTCAGTCCCTTGAGACTGCTG	8585[b]
F-SPEC	AACAGCTCTACCAGCTCTTTGCAAA	8720–8744
C-SPEC	AGACCCCAATACTGCACAAGACTT	8615–8638
5′E	CAGGAAAGGAATGAAAAGGATTTGTTA	8181–8207
3′E	ATAACCCTATCTGTCCACCCC	8693–8713
5′A	GANAACATGACCTGGCTGC	8094–8112
3′A	TCTATAACCCTATCTGTCCAGCCA	8693–8716
5′G	ACAATTACACATACCACATATACAGCC	8131–8757
3′G	TCTATAACCCTATCTGTCCAGTT	8694–8716
5′D	ACCACTAATGTGCCCTGGAACT	8037–8058
3′D	AGGAGGGTCTGAAATGACAGA	8356–8386
b-F	AGAGATGGCCACGGCTGCTT	
b-R	ATTTGCGGTGGACGATGGAG	

[a] HXB2 no. indicates primer position corresponding to nucleotide number of HXB2.
[b] HXB2 does not have sequence corresponding to primer, because of gap.

PCR of the v3 Region of the *gp120* Gene

The v3 region of HIV-1 provirus was amplified by nested PCR using primers 5′C2V3 and 3′V3 for the first round PCR reaction, and J5′-2KSI and 3′C2V3 for the second round reaction as described previously [Yagyu et al., 2002] (Table II). The PCR products were then subjected to 1% agarose gel electrophoresis at 100 V for 30 min, recovered from the gel, and used as a template for nucleotide sequencing.

Nucleotide Sequencing and Analysis

The sequencing reaction was carried out with a dideoxynucleotide cycle sequencing kit (Perkin Elmer, Wellesley, MA) using J5′-2KSI and 3′C2V3 as sense

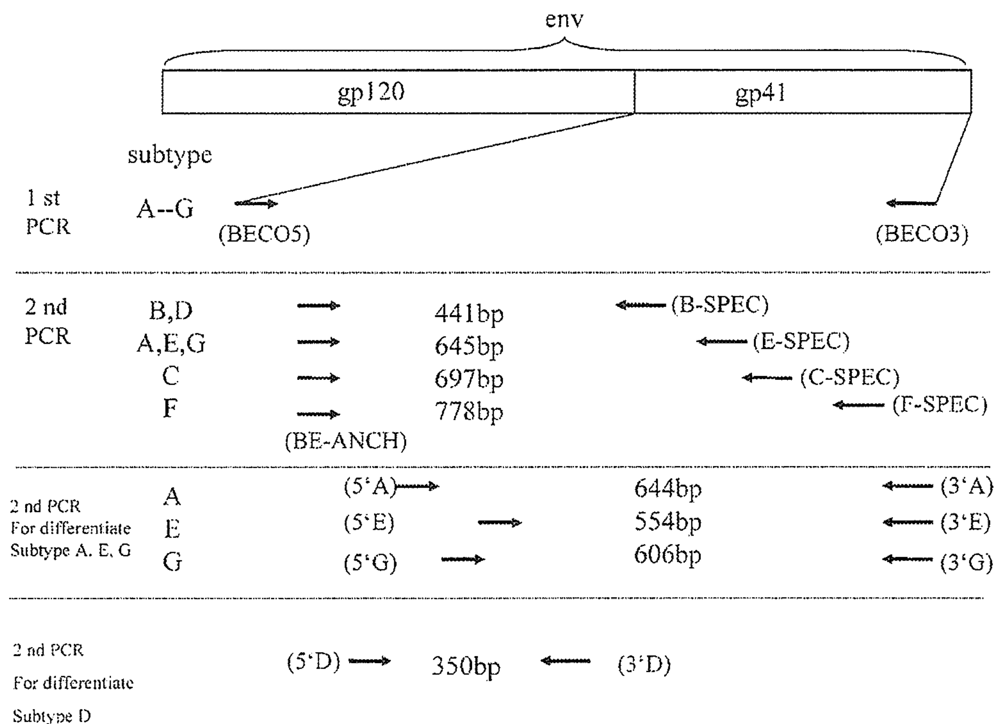

Fig. 1. Primer location and length of polymerase chain reaction (PCR) products.

and anti-sense primers for v3 region, respectively (Table II). The reaction products were analyzed by an automated DNA sequencer (ABI). The sequences of samples were aligned with references strains of subtypes A, B, C, D, F, G, CRF01_AE, and CRF02_AG, and SIV for analysis of v3 region by Clustal X [Thompson et al., 1997]. The subtypes were determined by phylogenetic trees, which were constructed using the neighbor-joining (NJ) method [Saitou and Nei, 1987].

RESULTS

Differentiation of Subtypes B and E by PCR With Subtype-Specific Primers

PCR to differentiate subtypes B and CRF01_AE was performed using samples from 33 subjects. A 441-bp PCR product, which was amplified between BE-ANCH and B-SPEC, was generated from the samples of 10 subjects, and a 645-bp PCR product, which was amplified between BE-ANCH and E-SPEC, was generated from the samples of 17 subjects. However, six subjects were negative, no PCR product was generated (Table I). All samples were positive for cellular beta-actin. This experiment was carried out to determine whether or not the primers B-SPEC and E-SPEC would react with the different subtypes. This information was considered useful for designing of new primers.

Determination of HIV-1 Subtype by Nucleotide Sequence Analysis of the v3 Region

In order to differentiate correct subtypes of the subjects, sequence analysis of the v3 region was performed, since this is considered the most reliable method. PCR of the v3 region of all samples generated successfully products of approximately 350 bp, which were then directly sequenced. The phylogenetic tree showed that the number of subjects of subtypes A, B, C, D, F, G, CRF01_AE, and CRF02_AG were 4, 6, 3, 6, 2, 3, 6, and 3, respectively (Tables I and III).

Designing of Env-Specific Primers for Differentiating Subtypes A, B, C, D, F, G, and CRF01_AE

For the first round PCR, the subtype-common primers, BECO5 and BECO3, were designed to differentiate subtypes B and CRF01_AE. Between BECO5 and BECO3, the subtype-unique conserved sequences of subtypes C and F were found, and used to design subtype C- and F-specific primers, respectively. However, the unique sequences specific for subtypes A, D, G, and CRF01_AE that could be used as anti-sense primers were not found. The results showed that the samples which were positive by PCR using BE-ANCH and subtype E-specific primers could be either subtype A, E, or G according to the sequence alignment, and this observation was in good agreement with a previous study [Yagyu et al., 2002]. Therefore, when the sample was positive for subtype A, E, or G by using the subtype E-specific primer, it was necessary to perform the second round PCR by using the first PCR product as the template with the alternative sets of subtype-specific primers, 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G (Table II, Fig. 2). Similarly, the sample which was positive for subtype B or D by using the subtype B-specific primer should be differentiated between subtypes B and D in the second round PCR using the first round PCR product as the template with the alternative set of primers 5′D and 3′D (Table II, Fig. 2).

Determination of Subtypes With Subtype-Specific Primers

The novel subtype-specific primers designed in this study were verified for specificity by testing with clinical specimens of the known subtypes. The primers used for the first round PCR were BECO5 and BECO3, and then the second round PCR was performed using the first round PCR product as the template, BE-ANCH as the 5′ primer and B-SPEC, C-SPEC, E-SPEC, and F-SPEC as the 3′ primers (Table II, Figs. 1, 2, and 3A). The primers C-SPEC and F-SPEC generated products of 675 and 778 bp, and the samples were identified as subtype C and F, respectively. A PCR product of 440 bp was also generated, possibly due to the annealing of the B-SPEC primer to the proviral DNA of subtypes B and D in the sample. Then, using the first PCR product as template, the second PCR was performed with 5′D and 3′D primers for detecting subtype D, and a 350-bp product was generated (Figs. 1, 2, and 3B). Similarly, E-SPEC annealed to subtypes A, E, and G, and generated a 650-bp product. Then, using the first PCR product as template, the second PCR was performed with a mixture of the primers 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G, and

TABLE III. Subtypes Determined by v3 Sequence and PCR Using Novel Primers

Subtype by v3 sequence	No. of samples with subtype by PCR using novel primers								
	A	B	C	D	F	G	CRF01_AE	ND	Total (n = 33)
A	3							1	4
B		5						1	6
C			3						3
D				5				1	6
F					2				2
G						3			3
CRF01_AE							6		6
CRF02_AG	2							1	3

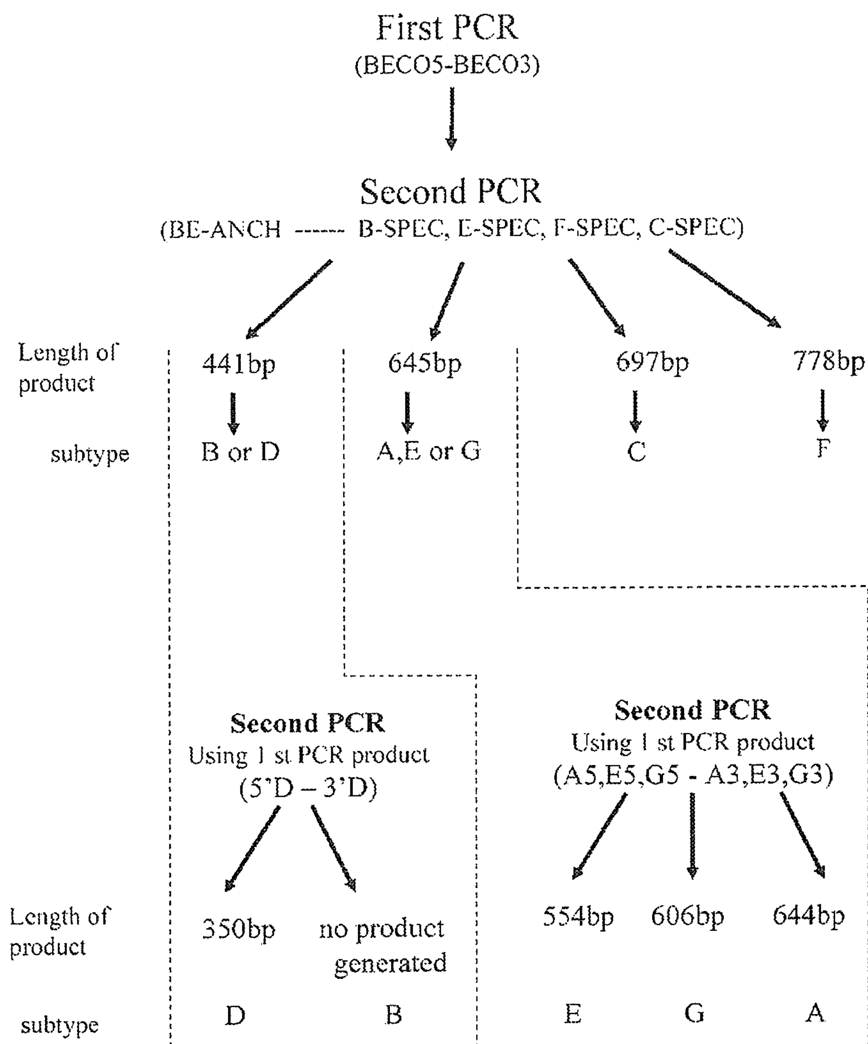

Fig. 2. Procedure of PCR for differentiation of subtypes with newly designed primers.

products of 644, 554, and 606 bp were generated for subtypes A, E, and G, respectively (Figs. 1, 2, and 3C). A total of 33 subjects were tested by this procedure using the newly designed primers. When BE-ANCH was used as a 5′ end primer in combination with B-SPEC, E-SPEC, C-SPEC, and F-SPEC as 3′ end primers, and the first round product was used as the template, PCR products of 441, 645, 697, and 778 bp were generated from 10, 14, 3, and 2 out of the 33 subjects, respectively (Tables I and III). In addition, when the alternative second round PCR for differentiation of subtypes A, G, and CRF01_AE was performed using the first round PCR product as a template and a mixture of the 5′A and 3′A, 5′E and 3′E, 5′B and 3′G primer pairs, 644 bp (subtype A), 554 bp (CRF01_AE), and 606 bp (subtype G) products were generated from 5, 6, and 2, out of the 13 subjects, respectively. Furthermore, when 5′D and 3′D were used in the alternative second round PCR to differentiate subtype D from subtype B, a 350 bp (subtype D) product was generated from 6 out of 12 subjects. The other 6 of the 12 subjects were therefore identified as subtype B.

DISCUSSION

The PCR for differentiation of subtype B and CRF01_AE was carried out as described previously [Yagyu et al., 2002]. The primers B-SPEC and E-SPEC have cross-reaction with other subtypes, except for subtype B and CRF01_AE. The B-SPEC primer anneals subtypes B and D, and the E-SPEC primer anneals subtypes A, C, G, and CRF01_AE. The result of PCR of cellular beta-actin was positive for all samples, suggesting that the DNA in samples was intact by the time of testing and the extraction procedure was successful. The samples that did not generate any products were considered not to have been amplified by the B-SPEC and E-SPEC primers, possibly because there was an insufficient copy number of the HIV-1 provirus or because it was subtype F [Yagyu et al., 2002].

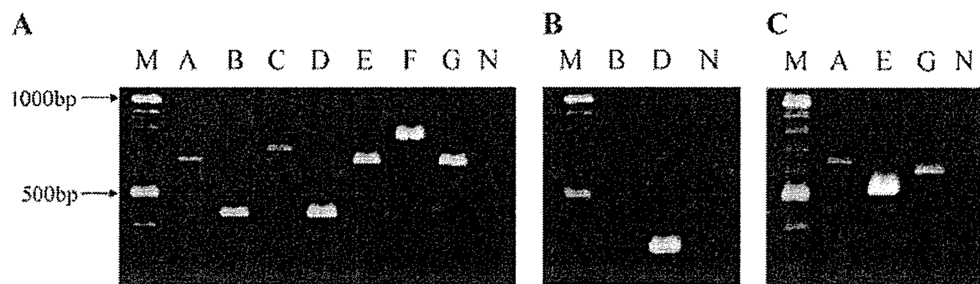

Fig. 3. Determination of subtypes with subtype-specific primers. **A**: PCR with primers (BECO5 and BECO3 for the first round PCR and, BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers C-SPEC and F-SPEC for the second round PCR) of subtype A (**lane A**), B (**lane B**), C (**lane C**), D (**lane D**), CRF01_AE (**lane E**), F (**lane F**), and G (lane F). PCR products were subjected to agarose gel electphoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**). **B**: PCR with primers (BECO5 and BECO3 for the first round PCR and, 5′D and 3′D for the second round PCR) of subtype B (**lane B**) and D (**lane D**). PCR products were subjected to agarose gel electrophoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**). **C**: PCR with primers (BECO5 and BECO3 for the first round PCR and, 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the second round PCR) of subtype A (**lane A**), CRF01_AE (**lane E**), and G (**lane G**). PCR products were subjected to agarose gel electphoresis with 100 bp ladder markers (**lane M**) and negative control (**lane N**).

Among the patients (BRON04, NG02, NG08, NG12, JP02, and KA18) who were negative for PCR for differentiation of subtypes B and E, BRON04 and KA18 were subtype F as expected. And, the subjects, NG02, NG08, NG12, and JP02, were subtypes A, CRF02_AG, D, and B, respectively.

The newly designed subtype-specific primers could be used as anti-sense primers against BE-ANCH, and could generate PCR products of different lengths. Specific primers for subtypes C and F which act as the anti-sense primers against BE-ANCH were also designed. However, it was rather difficult to design a subtype-specific for A, B, D, G, or CRF01_AE, because subtypes A, E, and G and subtypes B and D, respectively, were similar to each other. After the second PCR with primers BE-ANCH, B-SPEC, C-SPEC, E-SPEC, and F-SPEC, the sample which was positive for the subtype B-specific primer was further differentiated between subtypes B and D by using the subtype D-specific primers 5′D and 3′D. By this strategy, the primers were able to recognize only subtype D, since it was impossible to design a subtype B-specific primer for the second PCR. Since the subtypes B and D are very closely related, an attempt was made to differentiate the subsubtypes by PCR. The results showed that PCR was able to separate them incompletely in this experiment. However, because subtypes B and D are predominant in different countries, the differentiated subtypes B and D could provide important information on the course of an HIV strain newly imported to a particular country.

Three samples (OS08, NG06, NG08) of CRF02_AG determined by nucleotide sequence were differentiated as subtype A by PCR with the novel primers. The envelope gene of CRF02_AG consists of subtype A and G. Therefore, it may be impossible to design specific primers for CRF02_AG, when only the genome of gp41 is used for subtype determination.

The subtypes of all subjects determined by PCR using newly designed primers were in complete agreement with those determined by nucleotide sequence analysis of the v3 region except for CRF02_AG. Nevertheless, there were four patients, who did not generate any positive signals. This indicates that the relatively lower sensitivity of PCR with novel primers might not be due to the low copy number of proviral DNA; rather, it might be due to mismatched of the primer, since point mutations readily occur in the HIV-1 genome.

The newly designed primers functioned accurately and conclusively. In comparison with PCR as a method for the determination of subtypes, sequence analysis requires better-trained personnel, more expensive reagents, and more equipment and time. The PCR method is useful for developing countries in which the burden of HIV/AIDS has increased dramatically. However, although the PCR method has the above advantages, it also has a shortcoming in that it differentiates subtypes only in the gp41 region. In order to evaluate this method accurately, a larger number of samples should be tested.

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REFERENCES

Carr JK, Salminen MO, Koch C, Gotte D, Artenstein AW, Hegerich PA, St. Louis D, Burke DS, McCutchan FE. 1996. Full-length sequence and mosaic structure of a human immunodeficiency virus type 1 isolate from Thailand. J Virol 70:5935–5943.

Carr JK, Salminen MO, Albert J, Sanders-Buell E, Gotte D, Birx DL, McCutchan FE. 1998. Full genome sequences of human immunodeficiency virus type 1 subtypes G and A/G intersubtype recombinants. Virology 247:22–31.

Charneau P, Borman AM, Quillent C, Guetard D, Chamaret S, Cohen J, Remy G, Montagnier L, Clavel F. 1994. Isolation and envelope sequence of a highly divergent HIV-1 isolate: Definition of a new HIV-1 group. Virology 205:247–253.

Cheingsong-Popov R, Lister S, Callow D, Kaleebu P, Beddows S, Weber J. 1994. Serotyping HIV type 1 by antibody binding to the V3 loop: Relationship to viral genotype. WHO network for HIV isolation and characterization. AIDS Res Hum Retroviruses 10:1379–1386.

Delwart EL, Shpaer EG, Louwagie J, Mullins JI. 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: Analysis of *HIV-1 env* genes. Science 262:1257–1261.

Essex M. 1999. Human immunodeficiency viruses in the developing world. Adv Virus Res 53:71–88.

Gao F, Robertson DL, Morrison SG, Hui H, Craig S, Decker J, Fultz PN, Girard M, Shaw GM, Hahn BH, Sharp PM. 1996. The heterosexual human immunodeficiency virus type 1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. J Virol 70:7013–7029.

Gaywee J, Artenstein AW, VanCott TC, Trichavaroj R, Sukchamnong A, Amlee P, de Souza M, McCutchan FE, Carr JK, Markowitz LE, Michael R, Nittayaphan S. 1996. Correlation of genetic and serologic approaches to HIV-1 subtyping in Thailand. Acquir Immune Defic Syndr Hum Retrovirol 13:392–396.

Gurtler LG, Hauser PH, Eberle J, von Brunn A, Knapp S, Zekeng L, Tsague JM, Kaptue L. 1994. A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. J Virol 68: 1581–1585.

Hoelscher M, Dowling WE, Sanders-Buell E, Carr JK, Harris ME, Thomschke A, Robb ML, Birx DL, McCutchan FE. 2002. Detection of HIV-1 subtypes, recombinants, and dual infections in east Africa by a multi-region hybridization assay. AIDS 16:2055–2064.

Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC. 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73:152–160.

Loussert-Ajaka I, Chaix ML, Korber B, Letourneur F, Gomas E, Allen E, Ly TD, Brun-Vezinet F, Simon F, Saragosti S. 1995. Variability of human immunodeficiency virus type 1 group O strains isolated from Cameroonian patients living in France. J Virol 69:5640–5649.

Novitsky V, Arnold C, Clewley JP. 1996. Heteroduplex mobility assay for subtyping HIV-1: Improved methodology and comparison with phylogenetic analysis of sequence data. J Virol Methods 59:61–72.

Osmanov S, Pattou C, Walker N, Schwardlander B, Esparza J. 2002. Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000. J Acquir Immune Defic Syndr 29:184–190.

Ou CY, Takebe Y, Luo CC, Kalish M, Auwanit W, Bandea C, DeLaTorre N, Moore JL, Schochetman G, Yamazaki S, Gayle HD, Young NL, Weniger BG. 1992. Wide distribution of two subtypes of HIV-1 in Thailand. Aids Res Hum Retroviruses 8:1471–1472.

Ou CY, Takebe Y, Weniger BG, Luo CC, Kalish ML, Auwanit W, Yamazaki S, Gayle HD, Young NL, Schochetman G. 1993. Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. Lancet 341:1171–1174.

Palker TJ, Clark ME, Langlois AJ, Matthews TJ, Weinhold KJ, Randall RR, Bolognesi DP, Haynes BF. 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. Proc Natl Acad Sci USA 85:1932–1936.

Plantier JC, Vergne L, Damond F, MBoup S, MPoudi-NGole E, Buzelay L, Farfara I, Brand D, Peeters M, Brun-Vezinet F, Delaporte E, Barin F. 2002. Development and evaluation of a DNA enzyme immunoassay method for env genotyping of subtypes A through G of human immunodeficiency virus type 1 group M, with discrimination of the circulating recombinant forms CRF01_AE and CRF02_AG. J Clin Microbiol 40:1010–1022.

Robert-Guroff M, Louie A, Myagkikh M, Michaels F, Kieny MP, White-Scharf ME, Potts B, Grogg D, Reitz MS, Jr. 1994. Alteration of V3 loop context within the envelope of human immunodeficiency virus type 1 enhances neutralization. J Virol 68:3459–3466.

Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK, Gao F, Hahn BH, Kalish ML, Kuiken C, Learn GH, Leitner T, McCutchan F. 2000. HIV-1 nomenclature proposal. Science 288: 55–57.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Simon F, Mauclere P, Roques P, Loussert-Ajaka I, Muller-Trutwin MC, Saragosti S, Georges-Courbot MC, Barre-Sinoussi F, Brun-Vezinet F. 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. Nat Med 4:1032–1037.

Takahashi H, Cohen J, Hosmalin A, Cease KB, Houghten R, Cornette JL, DeLisi C, Moss B, Germain RN, Berzofsky JA. 1988. An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. Proc Natl Acad Sci USA 85:3105–3109.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882.

Triques K, Bourgeois A, Saragosti S, Vidal N, Mpoudi-Ngole E, Nzilambi N, Apetrei C, Ekwalanga M, Delaporte E, Peeters M. 1999. High diversity of HIV-1 subtype F strains in Central Africa. Virology 259:99–109.

Triques K, Bourgeois A, Vidal N, Mpoudi-Ngole E, Mulanga-Kabeya C, Nzilambi N, Torimiro N, Saman E, Delaporte E, Peeters M. 2000. Near-full-length genome sequencing of divergent African HIV type 1 subtype F viruses leads to the identification of a new HIV type 1 subtype designated K. AIDS Res Hum Retroviruses 16:139–151.

UNAIDS/WHO . 2002. AIDS epidemic update. December 2002.

Vanden Haesevelde M, Decourt JL, De Leys RJ, Vanderborght B, van der Groen G, van Heuverswijn H, Saman E. 1994. Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. J Virol 68:1586–1596.

Wasi C, Herring B, Raktham S, Vanichseni S, Mastro TD, Young NL, Rubsamen-Waigmann H, von Briesen H, Kalish ML, Luo CC, et al. 1995. Determination of HIV-1 subtypes in injecting drug users in Bangkok, Thailand, using peptide-binding enzyme immunoassay and heteroduplex mobility assay: Evidence of increasing infection with HIV-1 subtype E. AIDS 9:843–849.

Weniger BG, Brown T. 1996. The march of AIDS through Asia. N Engl J Med 335:343–345.

Yagyu F, Ikeda Y, Ariyoshi K, Sugiura W, Wongkhomthong SA, Masuda M, Ushijima H. 2002. Differentiation of subtypes B and E of human immunodeficiency virus type 1 by polymerase chain reaction using novel env gene primers. J Virol Methods 101: 11–20.

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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.
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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

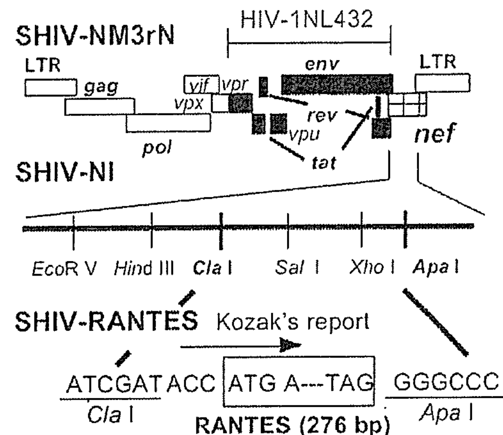

Fig. 1. Genetic structure of SHTV-RANTES. The *Cla* I and *Xho* I region of parental SHIV-NI, the *nef* cassette vector, was replaced by the human RANTES gene. The ORF including the initiation (ATG) and stop (TAG) codons of the RANTES gene is shown in the box. The flanking sequence of the RANTES initiation 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,

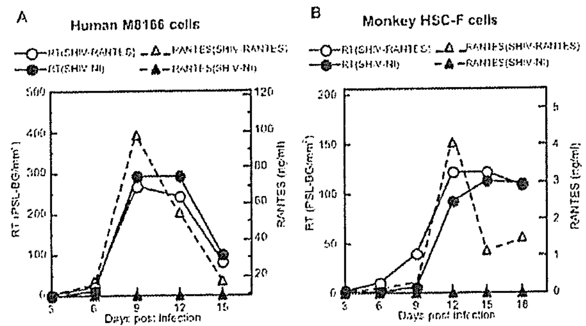

Fig. 2. Kinetics of virus replication and RANTES production with SHIV-RANTES and SHIV-NI in human (A) and monkey (B) CD4+ cell lines. Viral replication was monitored by RT activity in the supernatant of cell cultures infected with SHIV-RANTES (open circles) or SHIV-NI (closed circles). The unit for RT 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