J Med Primatol 2005: 34: 294–302

JOURNAL OF MEDICAL PRIMATOLOGY

Early virological events in various tissues of newborn monkeys after intrarectal infection with pathogenic simian human immunodeficiency virus

Miyake A, Ibuki K, Suzuki H, Horiuchi R, Saito N, Motohara M, Hayami M, Miura T. Early virological events in various tissues of newborn monkeys after intrarectal infection with pathogenic simian human immunodeficiency virus. J Med Primatol 2005; 34:294–302.

Abstract: Children infected with human immunodeficiency virus type 1 often have higher viral loads and progress to acquired immunodeficiency syndrome more rapidly than adults. In our previous study of simian–human immunodeficiency virus (SHIV)-infected adult monkeys, immature CD4CD8 double-positive T cells in the thymus and jejunum decreased faster than mature CD4 single-positive T cells. Here, we examined the effect of virus replication on immature T cells from the same SHIV-inoculated newborn monkeys having more immature T cells than adults. The infectious viruses were more abundantly detected in the thymus than in other tissues at both 13 and 26 days post-infection (dpi). However, mature $CD4^+$ T cells in the thymus declined after 13 dpi and immature $CD3^-$ CD4 single-positive T cells remained at 26 dpi. These results suggested that many immature $CD4^+$ T cells in the thymus of newborns support the production of infectious viruses even after the depletion of mature $CD4^+$ T cells.

Ariko Miyake, Kentaro Ibuki, Hajime Suzuki, Reii Horiuchi, Naoki Saito, Makiko Motohara, Masanori Hayami, Tomoyuki Miura

Institute for Virus Research, Kyoto University, Kyoto, Japan

Key words: early phase of infection – immature T cells – newborn monkey – SHIV – thymus

Accepted April 25, 2005.

Tomoyuki Miura DVM PhD, Laboratory of Primate Model, Experimental Research Center for Infectious Disease, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto, 606-8507, Japan. Tel.: 81 75 751 3981; fax: 81 75 761 9335; e-mail: tmiura@virus.kyoto-u.ac.jp

Funding: This work was supported by a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan and a Grant-in-Aid for Scientific Research from the Ministry of Education and Science, Japan. A. M. is a recipient of a Research Resident Fellowship for junior researchers of the Japanese Foundation for AIDS Prevention.

Introduction

Children infected with human immunodeficiency virus type 1 (HIV-1) often have higher viral loads and progress to AIDS more rapidly than adults [2, 8, 20]. In the absence of antiretroviral treatments, the disease progresses rapidly, with up to 45% of infected children developing AIDS and dying within the first 2 years of life [Joint United Nations Programme on HIV/AIDS (UNAIDS), 2002], while adults with HIV-1 infection have an asymptomatic period for 10 or more years. The reason for the rapid progression and high viral loads in newborns is unclear. The advanced stage of disease has generally been attributed to the immaturity of the immune system. However, children are reported to have active cellular and humoral immune responses to HIV infection [12, 23]. Moreover, in a study using the simian immunodeficiency virus (SIV)-macaque monkey model, Veazey et al. [22] showed that newborn monkeys have more activated T cells in the intestinal tract and that activated cells were the source of high viral load and the rapid progression to AIDS.

In our previous study using adult macaque monkeys, we observed that the percentage of CD4CD8 double positive (DP) T cells was much higher in the thymus and intestinal tract than in other tissues examined [Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, Suzuki M, Saito N, Nakasone T, Honda M, Watanabe T, Miura T, Hayami M, unpublished data]. Both tissues were reported as sites of maturation of lymphocytes [4, 10], and CD4CD8 DP T cells have been proposed to represent immature stages in T cell development. These immature CD4CD8 DP T cells decreased earlier than mature CD4 single positive (SP) T cells in the thymus and jejunum after infection of a

pathogenic SIV/HIV-1 chimeric virus [simian–human immunodeficiency virus (SHIV)]. These results suggested that the rate of virus replication is greater in immature T cells than in mature T cells. Newborns have more immature T cells than adults [5, 13], which might contribute to the higher rate of disease progression in newborns.

In this study, we analyzed newborn monkeys infected with the SHIV that were used in the study with adult monkeys [Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, Suzuki M, Saito N, Nakasone T, Honda M, Watanabe T, Miura T, Hayami M, unpublished data], and compared the results in newborn and adult monkeys. In newborns examined at both 13 and 26 days after inoculation, infectious viruses were more abundant in the thymus than in other tissues. In the lymphoid tissues except the thymus, $CD4^+$ T cells were more rapidly and profoundly depleted in newborns than in adults. Moreover, not many $CD4^+$ T cells were present in the intestinal tracts of newborns, even in the uninfected control. However, in the thymus of newborns, there were many $CD4^+$ T cells including mature and immature $CD4^+$ T cells at 13 days after inoculation, and immature $CD3^-$ $CD4^+$ T cells remained even after the depletion of mature $CD4^+$ T cells.

Materials and Methods

Virus

The SHIV-C2/1 was generated by *in vivo* passage of SHIV-89.6 (containing env, tat, rev and vpu derived from primary isolates of HIV-1) [17]. SHIV-C2/1 KS661c is a molecular clone, which was constructed from the consensus sequence of SHIV-C2/1 (GenBank accession number AF217181). SHIV-C2/1 KS661c can infect macaque monkeys by intravenous and intrarectal routes and cause precipitous viremia and drastic $CD4^+$ cells depletion. The virus stock was prepared from supernatant of a human lymphoid cell line, CEMx174, and stored in liquid nitrogen (−190°C) until use. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus stock was measured in CEMx174. Twenty $TCID_{50}$ was equivalent to one 50% macaque infectious dose (MID_{50}).

Monkeys and virus inoculation

Three newborn rhesus macaques (*Macaca mulatta*) were used in this study. All monkeys used in this study were treated in accordance with the institutional regulations approved by the Committee for Experimental Use of Nonhuman Primate in the Institute for Virus Research, Kyoto University. Two monkeys were anesthetized by intramuscular injection of ketamine chloride and inoculated intrarectally with 2×10^3 $TCID_{50}$ of SHIV-C2/1 KS661c. All intrarectal inoculations were performed with a pediatric feeding catheter inserted a distance of 5 cm from the anus. The catheter was inserted carefully to avoid causing trauma. The inoculated monkeys were killed at 13 and 26 days after inoculation, respectively. The third monkey was not inoculated and was used as a normal control.

Sample collection

Prior to killing, blood was collected from the inguinal vein under ketamine anesthesia. Intravenous pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL, USA) (40 mg/kg) was administered for deeper anesthesia. After thoracotomy, the right atrium was incised, and 500 ml of sterile heparinized saline (5 U/ml) was infused into the left ventricle using an 18-gauge needle attached to infusion tubing. After the perfusion, complete sets of organs were obtained for the following analyses. Peripheral blood mononuclear cells (PBMC) and plasma were separated from heparinized blood by Percoll (Lymphocyte Separation Solution; Nacalai Tesque, Kyoto, Japan) density gradient centrifugation. Plasma was frozen at −80°C until used. Parts of the organ samples were frozen directly at −80°C until used for quantification of proviral DNA. Residual samples of spleen, thymus and axillary, inguinal and mesenteric lymph nodes were minced and filtered through a 40 μm nylon filter (Becton Dickinson, Franklin Lakes, NJ, USA). Samples of jejunum were washed in Dulbecco's Modified Eagle's Medium (DMEM) containing 0.45 mM DTT, cut into 1-cm^2 pieces and agitated in DMEM medium containing 5% fetal calf serum (FCS) for 1 hour at room temperature (RT). After a short sedimentation, supernatants and tissue fragments were processed to give intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL), respectively. The supernatants (containing IEL) were filtered through columns containing packed glass wool and centrifuged at 1600 rpm for 7 minute. The pellets were suspended in 30% Percoll (Pharmacia, Uppsala, Sweden) and centrifuged at 1800 rpm for 20 minute. The pellets were resuspended in 44% Percoll, layered on 70% Percoll and centrifuged at 1800 rpm for 20 minute. Cells at the interface between the 44 and 70% Percoll layers were collected. The residual tissue fragments were agitated in Hanks buffer containing 5 mM EDTA for 10 minutes at RT, and the supernatants were removed. This step was repeated three times. The

fragments were suspended in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% FCS, and after agitation for 30 minutes at RT the supernatants were removed. The fragments were resuspended in RPMI 1640 medium containing 10% FCS and 0.2 mg/ml of collagenase (type II; Sigma, St Louis, MO, USA) and agitated for 90 minutes at RT. The suspensions (containing LPL) were filtered through the glass wool columns, and cells were enriched by Percoll density gradient centrifugation as described above for IEL. The cells obtained from each organ were immediately used in the infectious plaque assay and flow cytometry analysis.

Quantification of plasma viral RNA

Viral RNA loads in plasma were determined by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) [19]. Total RNA was prepared from plasma with a QIAamp Viral RNA kit (QIAGEN, Hilden, Germany). RT-PCR was performed with a Taqman EZ RT-PCR kit (Perkin Elmer, Wellesley, MA, USA) for the SIV gag region using the following primers: SIV2-696F (5′-GGA AAT TAC CCA GTA CAA CAA ATA GG-3′) and SIV2-784R (5′-TCT ATC AAT TTT ACC CAG GCA TTT A-3′). A labeled probe, SIV2-731T (5′-Fam-TGT CCA CCT GCC ATT AAG CCC G-Tamra-3′; Perkin Elmer), was used for detection of the PCR products. These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and analyzed using the manufacturer's software. For each run, a standard curve was generated from dilutions whose copy numbers were known, and the RNA in the plasma samples was quantified based on the standard curve.

Quantification of proviral DNA

The proviral DNA loads in tissues were determined by quantitative PCR. DNA samples were extracted directly from frozen tissues with a QIAGEN DNeasy Tissue kit (QIAGEN). PCR was performed with a Taqman PCR Reagent kit (Perkin Elmer) using the same primer set and probe that were used in RT-PCR. A standard curve was generated from a plasmid DNA sample containing the full genome of SHIV-NM-3rN, which was quantified with a UV-spectrophotometer.

Infectious plaque assay

Infectious viruses were quantified and isolated using the infectious plaque assay [6, 11]. An agarose gel bilayer containing RPMI 1640 medium was made in plastic culture dishes of 100 mm diameter; the lower layer consisted of 12 ml of 1.2% agarose (Agarose NA; Pharmacia) and the upper layer consisted of 12 ml of 0.4% low-gelling-temperature agarose (SeaPlaque Agarose; FMC, Rockland, ME, USA). The dishes were incubated at 37°C in 5% CO_2 overnight. The following day, 2×10^6 cells of each sample and 8×10^6 cells of M8166 were suspended in 3 ml of a 0.4% low-gelling-temperature agarose solution containing the culture medium and the mixture was immediately overlaid on the agarose gel layer previously prepared. After the gel was hardened, the plates were covered with 12 ml of culture medium and incubated at 37°C in 5% CO_2 for 10 days. The medium over the plates was replaced with fresh medium every day. After removal of the medium in the plates at 10 days, the plates were stained with 2 ml of 0.7% MTT for 2 hour to count the number of plaques.

Flow cytometry

The frequency of CD4 single-positive and CD4CD8 double-positive T cells in PBMCs and the various tissues were examined by flow cytometry. Lymphocytes were treated with anti-CD3 (FN-18-FITC; Biosource, Camarillo, CA, USA), anti-CD4 (Nu-TH/I-PE; NICHIREI, Tokyo, Japan) and anti-CD8 (SK1; Becton Dickinson Biosciences) monoclonal antibody and examined on a FACScan analyzer (Becton Dickinson Biosciences). The absolute number of lymphocytes in the blood was determined by using an automated blood cell counter (F-820; Sysmex, Kobe, Japan).

Results

Intrarectal infection of newborn macaques with SHIV-C2/1 KS661c

Two newborn rhesus macaque monkeys were intrarectally inoculated with SHIV- C2/1 KS661c and were killed at 13 and 26 days after inoculation, respectively. The plasma viral RNA load was about $1–3 \times 10^8$ copies/ml at 13 or 14 days after inoculation, and then decreased slightly but remained at 5×10^6 copies/ml at 26 days after inoculation (Fig. 1). These levels were almost the same as the levels we measured in adult monkeys in our previous study [Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, Suzuki M, Saito N, Nakasone T, Honda M, Watanabe T, Miura T, Hayami M, unpublished data]. In the adults, the plasma viral RNA load peaked at about 10^8 to 5×10^9 copies/ml

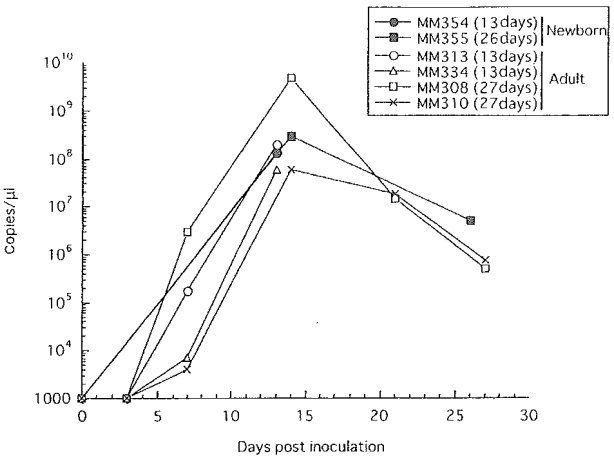


Fig. 1. Plasma viral RNA loads of two newborn and four adult monkeys intrarectally inoculated with SHIV-C2/1-KS661c as determined by quantitative RT-PCR. The detection limit of this assay was 1×10^3 copies/ml.

at 13 days after inoculation, and remained 5×10^5 to 10^6 copies/ml at 27 days after inoculation (Fig. 1). Before the inoculation, $CD4^+$ T cell counts in peripheral blood in the newborn monkeys (1700–2500 cells/μl) were higher than those in the adult monkeys (500–1200 cells/μl; Fig. 2). However, the $CD4^+$ T cell count in newborn monkeys dropped to <500 cells/μl by 13 or 14 days after inoculation, which is the same level as was observed in the adult monkeys, and remained at this low level until 26 days after inoculation.

Detection of proviral DNA in various tissues soon after intrarectal inoculation

To investigate viral distribution to the systemic tissues early after mucosal infection, proviral DNAs

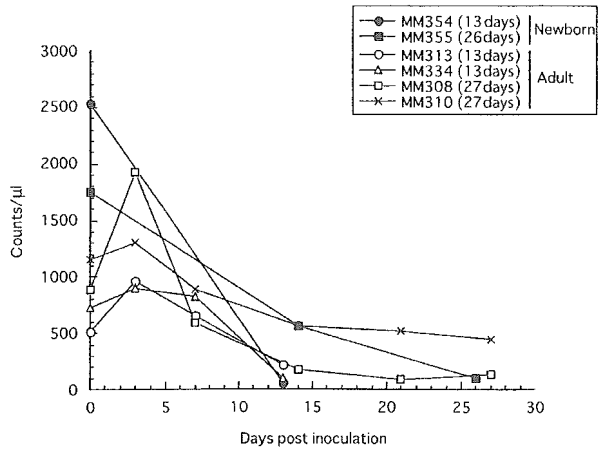


Fig. 2. Number of $CD4^+$ T cells in peripheral blood of two newborn and four adult monkeys inoculated with SHIV-C2/1-KS661c.

in various tissues were quantified by quantitative PCR. In the monkey that was examined at 13 days after inoculation (MM354), proviral DNA was detected in all the tissues examined at high levels (Fig. 3). The levels of proviral DNA in the PBMCs, spleen, lymph nodes and intestinal tract were each about 10^3 to 10^4 copies/μg. The level of proviral DNA in the thymus (10^5 copies/μg) was about 10 times higher than the levels in the other tissues examined. The levels of proviral DNA in the non-lymphoid tissues, including lung, liver, kidney and brain were about 20 to 2×10^3 copies/μg (data not shown). The titers of proviral DNA remained high in all the tissues of the monkey examined at 26 days after inoculation (MM355; Fig. 3). These results indicated that the viral loads in various tissues were amplified within 13 days after inoculation and that the virus stocks remained in each tissue until 26 days after inoculation. Similar results were obtained for the adult monkeys at 13 and 27 days after inoculation (MM313 and MM310; Fig. 3). High titers of proviral DNA were detected in all examined tissues of adult monkeys at both 13–27 days after inoculation.

Detection of infectious virus in various tissues early after intrarectal infection

Although proviral DNA was present in each tissue early after the mucosal infection, it was not clear whether these tissues released infectious viruses, which are considered to play a major role in the spread of virus in the body. To observe the release of infectious viruses from the various tissues, we quantified the infectious viruses in those tissues using the plaque assay. As shown by plaque assays, infectious viruses were more abundantly in the thymus than that in the other tissues (Fig. 4). At 13 days after inoculation, 170 plaque forming units (pfu)/10^6 cells were detected in the thymus. This high titer of infectious virus remained until 26 days after inoculation (220 pfu/10^6 cells). However, in the other lymphoid tissues examined, the titers at 13 and 26 days after inoculation were <63 and 55.5 pfu/10^6 cells, respectively. In contrast, the level of infectious viruses in the lymphoid tissues of the adult monkeys were high at 13 days after inoculation, and those infectious viruses decreased profoundly by 27 days after inoculation (Fig. 4). However, the level of infectious viruses in the intestinal tract (jejunum IEL and LPL) were low in both the newborn and adult monkeys. In the newborn monkeys, the titers of infectious viruses in the intestinal tracts at 13 and 26 days after inoculation were <7.5 pfu/10^6 cells and <2 pfu/10^6 cells, respectively (Fig. 4), while in the adult

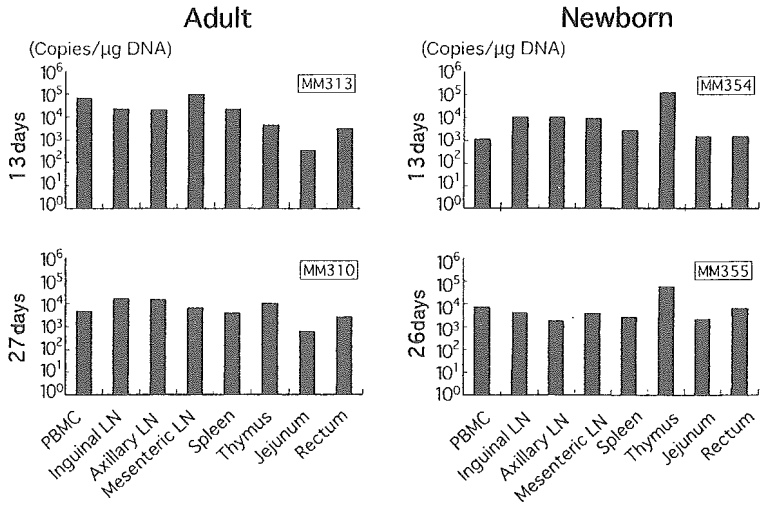


Fig. 3. Proviral DNA loads in various tissues of newborn and adult monkeys inoculated with SHIV-C2/1-KS661c. Viral loads were determined by quantitative PCR and are expressed as viral DNA copy numbers per microgram of total DNA extracted from tissue homogenates.

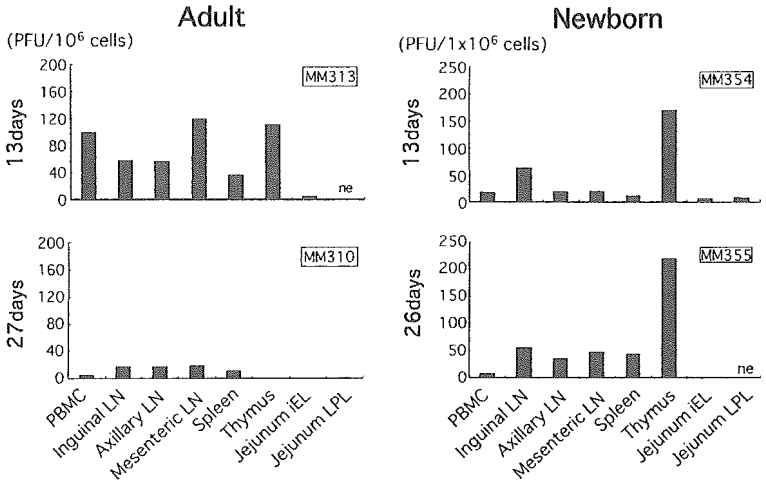


Fig. 4. Infectious virus loads in various tissues of newborn and adult monkeys inoculated with SHIV-C2/1-KS661c. Viral loads were determined by infectious plaque assay and are expressed as the number of plaque forming units (pfu) per 10^6 cells. 'ne' means the culture was not evaluated because of contamination.

monkeys, they were not detectable throughout the infection.

Sequential changes in the proportion of $CD4^+$ T cells in thymus

Infectious viruses were detected at extremely high levels in the thymus at 13 and 26 days after inoculation. The extent of virus replication in a tissue might be correlated with the proportion of $CD4^+$ T cells in the tissue, as $CD4^+$ T cells have been reported to be the main target and source for amplification of HIV, SIV and SHIV [1, 7, 14, 16]. Therefore, sequential changes in the proportion of $CD4^+$ T cells in the thymus were examined using flow cytometry. The thymus is a site of T cell development, in which T cells progress through four stages in the following order: CD3 negative ($CD3^-$) CD4 SP, $CD3^-$ CD4CD8 DP, CD3 positive ($CD3^+$) CD4CD8 DP, and $CD3^+$ CD4 SP. As shown by flow cytometry, the thymuses of both uninoculated adult and newborn monkeys had many more immature $CD3^-$ CD4CD8 DP T cells (white bars in Fig. 5A) and $CD3^+$ CD4CD8 DP T cells (light gray bars) than mature $CD3^+$ CD4 SP T cells (black bars; Fig. 5A). The percentage of $CD3^-$ CD4 SP T cells (hatched bars) were less in both uninoculated adults and newborns. In the adult monkeys, total $CD4^+$ T cells

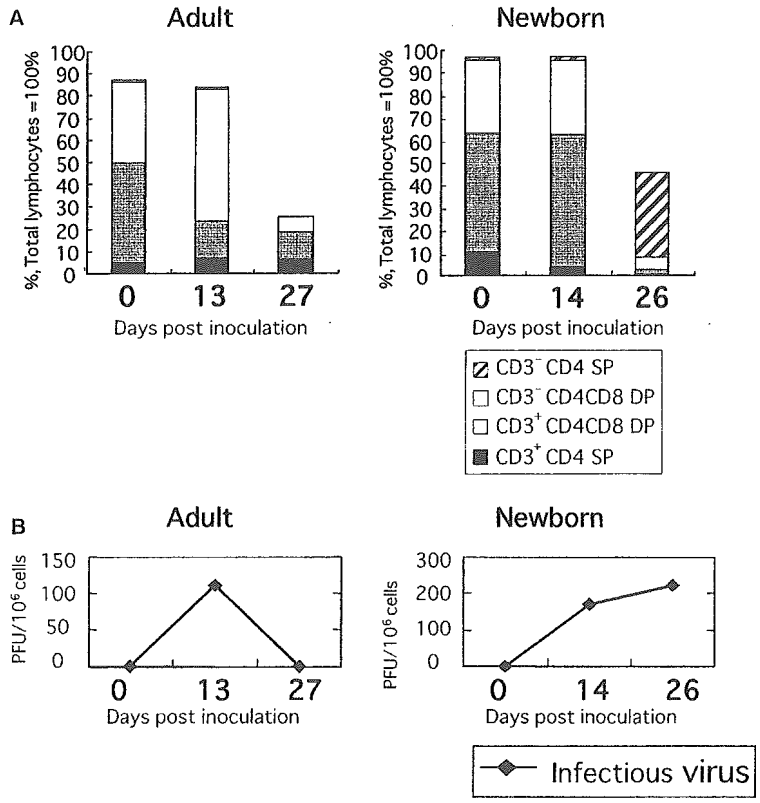


Fig. 5. (A) Changes in the proportions of $CD4^+$ T cells in the thymus of uninoculated and SHIV-inoculated monkeys. The percentage of $CD3^-$ CD4 single positive (SP), $CD3^-$ CD4CD8 double positive (DP), $CD3^+$ CD4CD8 DP, and $CD3^+$ CD4 SP cells in total lymphocytes was determined by flow cytometry. (B) Changes in the amount of infectious virus in the thymus of uninoculated and SHIV-inoculated monkeys.

(Fig. 5A) and the number of infectious viruses (Fig. 5B) decreased concurrently between 13 and 27 days after inoculation. In the newborn monkeys, mature $CD3^+$ CD4 SP T cells (black bars in right panel of Fig. 5A) and immature $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP T cells (white bars and light gray bars, respectively) also decreased between 13 and 26 days after inoculation. However, immature $CD3^-$ CD4 SP cells (hatched bars) were still present at 26 days after inoculation. The thymus of newborn monkeys also had high levels of infectious viruses at 26 days after inoculation (Fig. 5B, right panel). These results suggest that the immature $CD3^-$ CD4 SP T cells in the thymus of newborn monkeys were not destroyed by the virus infection and continued to produce infectious viruses.

Sequential changes in the proportion of $CD4^+$ T cells in various tissues

Sequential changes in the proportion of $CD4^+$ T cells were also examined in various tissues other than the thymus. The lymphoid tissues of the uninfected newborn had more $CD4^+$ T cells than those of adult monkeys (Fig. 6). In the newborn, these cells were already depleted at 13 days after inoculation. The decrease of $CD4^+$ T cells in the adult monkeys was more moderate and some of them remained even at 27 days after inoculation. On the contrary, the intestinal tracts of both the uninfected and virus-infected newborn monkeys did not have many $CD4^+$ T cells (Fig. 6). This suggests that the intestinal tracts of the newborn monkeys are not a major source for virus replication.

Discussion

Here, we show that, in newborn monkeys, the thymus is the main site of virus replication early after mucosal infection and that this replication was sustained by the immature $CD4^+$ T cells in the thymus. In the newborn monkey examined 13 days after intrarectal inoculation, the infectious viruses were detected more abundantly in the thymus than in the other tissues. Moreover, this high titer of infectious virus in the thymus was also present in the monkey examined 26 days after inoculation. These results were different from the results obtained with adult monkeys, which were analyzed

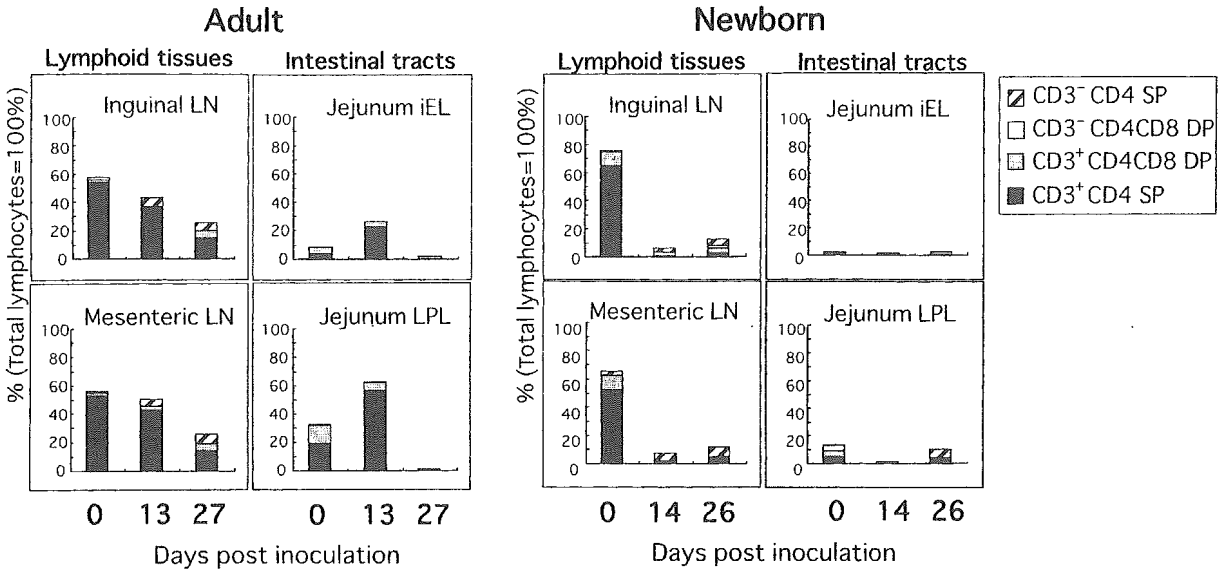


Fig. 6. Changes in the proportions of $CD4^+$ T cells in the lymphoid tissues and intestinal tracts of uninoculated and SHIV-inoculated monkeys. The percentage of $CD3^-$ CD4 single positive (SP), $CD3^-$ CD4CD8 double positive (DP), $CD3^+$ CD4CD8 DP, and $CD3^+$ CD4 SP cells in total lymphocytes was determined by flow cytometry.

in our previous research [Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, Suzuki M, Saito N, Nakasone T, Honda M, Watanabe T, Miura T, Hayami M, unpublished data]. First, in the adult monkeys examined at 13 days after inoculation, the high levels of infectious virus were detected not only in the thymus but also in the other lymphoid tissues. These differences between adult and newborn monkeys might result from differences in the proportion of $CD4^+$ T cells in total lymphocytes of each tissue of newborn and adult monkeys. $CD4^+$ T cells have been reported as the main target and source for amplification of HIV, SIV and SHIV [1, 7, 14, 16]. Therefore, the percentage of remaining $CD4^+$ T cells might be correlate with the level of virus produced there, whereas the decrease of $CD4^+$ T cells might reflect the extent of virus replication. In the adult monkeys, the proportion of $CD4^+$ T cells in total lymphocytes of all the lymphoid tissues slightly decreased after virus infection but some of them remained until 13 days after inoculation. However, in the newborn monkey examined at 13 days after inoculation, the $CD4^+$ T cells in each of the lymphoid tissues except the thymus were already depleted. The rapid depletion of $CD4^+$ T cells in newborn monkeys might explain the rapid progression to disease in newborn monkeys. However, the reason for the depletion of $CD4^+$ T cells is not clear because there are no data for the first 12 days after inoculation. Moreover, the small proportion of $CD4^+$ T cells in the lymphoid tissues of newborn monkey at 13 days after inoculation might explain the low titers of infectious virus that were observed in these tissues. In contrast, the thymus of newborn monkeys, like the thymus of adult monkeys, had many $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP cells in addition to $CD3^+$ CD4 SP cells. In addition, the proportion of whole $CD4^+$ cells in the thymus of the newborn monkeys at 13 days after inoculation was similar to that in the uninfected newborn monkey. $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP cells have been proposed to represent the immature stages in T cell development, whereas $CD3^+$ CD4 SP cells are regarded as mature $CD4^+$ T cells [3, 9]. These immature T cells were also reported to be susceptible to HIV-1 [15, 18, 21]. Therefore, the number of $CD4^+$ T cells including immature cells in the thymus of newborn monkey at 13 days after inoculation appeared to be sufficient to account for the high titers of infectious virus found in the thymus.

Infectious viruses in the thymus of the newborn monkey examined at 26 days after inoculation were also at a high titer. However, the infectious viruses in the lymphoid tissues including thymus of the adult monkeys profoundly decreased by 27 days after inoculation. In the adults, the proportion of $CD4^+$ T cells also decreased significantly in these lymphoid tissues by 27 days after inoculation. The decrease occurred in not only mature $CD3^+$ CD4 SP T cells but also immature $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP T cells in the thymus of the adults. A similar decrease

was detected in the thymus of the newborn monkey examined at 26 days after inoculation. However, $CD3^-$ CD4 SP cells, which represent a more immature stage of T cell development than $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP T cells, were still present in the thymus of the newborn monkey examined at 26 days after inoculation. Like immature $CD3^-$ CD4CD8 DP and $CD3^+$ CD4CD8 DP T cells, immature $CD3^-$ CD4 SP T cells were reported to be susceptible to HIV-1 [18]. Therefore, the immature $CD3^-$ CD4 SP cells in the thymus of newborn monkeys did not appear to decrease in number by the virus infection even after the depletion of mature $CD4^+$ T cells and appeared to sustain production of infectious viruses.

In the adults, the proportion of immature CD4CD8 DP T cells decreased earlier than that of mature CD4 SP T cells in the thymus after virus infection [Miyake A, Ibuki K, Enose Y, Suzuki H, Horiuchi R, Suzuki M, Saito N, Nakasone T, Honda M, Watanabe T, Miura T, Hayami M, unpublished data]. However, a similar decrease was not detected in the newborns. Because the thymus of newborns is more active than it is in adults, and has a larger potential for *de novo* generation of T cells than that in adults, it may be able to more readily supply T cells to replace those lost by the virus infection. Moreover, because immature $CD4^+$ T cells are just as susceptible to virus infection as mature $CD4^+$ T cells, the thymus of newborns may produce the virus more abundantly than the thymus of adults. In our study, the thymus of newborn monkeys had a significantly larger number of infectious viruses than the thymus of adult monkeys. These infectious viruses might eventually cause the rapid progression to disease.

Acknowledgments

We thank James Raymond for editing this manuscript.

References

1. Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA: The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312:763–767, 1984.
2. de Martino M, Tovo PA, Balducci M, Galli L, Gabiano C, Rezza G, Pezzotti P: Reduction in mortality with availability of antiretroviral therapy for children with perinatal HIV-1 infection. Italian Register for HIV Infection in Children and the Italian National AIDS Registry. JAMA 284:190–197, 2000.
3. Galy A, Verma S, Barcena A, Spits H: Precursors of CD3+CD4+CD8+ cells in the human thymus are defined by expression of CD34. Delineation of early events in human thymic development. J Exp Med 178:391–401, 1993.
4. Haynes BF, Denning SM, Le PT, Singer KH: Human intrathymic T cell differentiation. Semin Immunol 2:67–77, 1990.
5. Hirokawa K, Utsuyama M, Kasai M, Kurashima C: Aging and immunity. Acta Pathol Jpn 42:537–548, 1992.
6. Kato S, Hiraishi Y, Nishimura N, Sugita T, Tomihama M, Takano T: A plaque hybridization assay for quantifying and cloning infectious human immunodeficiency virus type 1 virions. J Virol Methods 72:1–7, 1998.
7. Klatzmann D, Champagne E, Chamaret S, Gruest J, Guetard D, Hercend T, Gluckman JC, Montagnier L: T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 312:767–768, 1984.
8. Kourtis AP, Nesheim SR, Thea D, Ibegbu C, Nahmias AJ, Lee FK: Correlation of virus load and soluble L-selectin, a marker of immune activation, in pediatric HIV-1 infection. AIDS 14:2429–2436, 2000.
9. Kraft DL, Weissman IL, Waller EK: Differentiation of CD3-4-8- human fetal thymocytes in vivo: characterization of a CD3-4+8- intermediate. J Exp Med 178:265–277, 1993.
10. Lundqvist C, Baranov V, Hammarstrom S, Athlin L, Hammarstrom ML: Intra-epithelial lymphocytes. Evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. Int Immunol 7:1473–1487, 1995.
11. Miyake A, Enose Y, Ohkura S, Suzuki H, Kuwata T, Shimada T, Kato S, Narayan O, Hayami M: The quantity and diversity of infectious viruses in various tissues of SHIV-infected monkeys at the early and AIDS stages. Arch Virol 149:943–955, 2004.
12. Pikora CA, Sullivan JL, Panicali D, Luzuriaga K: Early HIV-1 envelope-specific cytotoxic T lymphocyte responses in vertically infected infants. J Exp Med 185:1153–1161, 1997.
13. Ritter MA, Palmer DB: The human thymic microenvironment: new approaches to functional analysis. Semin Immunol 11:13–21, 1999.
14. Sattentau QJ, Clapham PR, Weiss RA, Beverley PC, Montagnier L, Alhalabi MF, Gluckmann JC, Klatzmann D: The human and simian immunodeficiency viruses HIV-1, HIV-2 and SIV interact with similar epitopes on their cellular receptor, the CD4 molecule. AIDS 2:101–105, 1988.
15. Schnittman SM, Denning SM, Greenhouse JJ, Justement JS, Baseler M, Kurtzberg J, Haynes BF, Fauci AS: Evidence for susceptibility of intrathymic T-cell precursors and their progeny carrying T-cell antigen receptor phenotypes TCR alpha beta + and TCR gamma delta + to human immunodeficiency virus infection: a mechanism for CD4+ (T4) lymphocyte depletion. Proc Natl Acad Sci U S A 87:7727–7731, 1990.
16. Shibata R, Kawamura M, Sakai H, Hayami M, Ishimoto A, Adachi A: Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J Virol 65:3514–3520, 1991.
17. Shinohara K, Sakai K, Ando S, Ami Y, Yoshino N, Takahashi E, Someya K, Suzaki Y, Nakasone T, Sasaki Y, Kaizu M, Lu Y, Honda M: A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J Gen Virol 80:1231–1240, 1999.
18. Su L, Kaneshima H, Bonyhadi M, Salimi S, Kraft D, Rabin L, McCune JM: HIV-1-induced thymocyte depletion is associated with indirect cytopathogenicity and

infection of progenitor cells in vivo. Immunity 2:25–36, 1995.
19. Suryanarayana K, Wiltrout TA, Vasquez GM, Hirsch VM, Lifson JD: Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. AIDS Res Hum Retroviruses 14:183–189, 1998.
20. Tovo PA, de Martino M, Gabiano C, Cappello N, D'Elia R, Loy A, Plebani A, Zuccotti GV, Dallacasa P, Ferraris G, Caselli D, Fundaro C, D'Argenio P, Galli L, Principi N, Stegagno M, Ruga E, Palomba E and the Italian Register for HIV Infection in Children. Prognostic factors and survival in children with perinatal HIV-1 infection. The Italian Register for HIV Infections in Children. Lancet 339:1249–1253, 1992.
21. Valentin H, Nugeyre MT, Vuillier F, Boumsell L, Schmid M, Barre-Sinoussi F, Pereira RA: Two subpopulations of human triple-negative thymic cells are susceptible to infection by human immunodeficiency virus type 1 in vitro. J Virol 68:3041–3050, 1994.
22. Veazey RS, Lifson JD, Pandrea I, Purcell J, Piatak M Jr, Lackner AA: Simian immunodeficiency virus infection in neonatal macaques. J Virol 77:8783–8792, 2003.
23. Weiblen BJ, Lee FK, Cooper ER, Landesman SH, McIntosh K, Harris JA, Nesheim S, Mendez H, Pelton SI, Nahmias AJ, *et al.*: Early diagnosis of HIV infection in infants by detection of IgA HIV antibodies. Lancet 335:988–990, 1990.

Tissue Antigens ISSN 0001-2815

Analysis of evolutionary conservation in CD1d molecules among primates

N. Saito[1,2], M. Takahashi[1,3], W. Akahata[2], E. Ido[2], C. Hidaka[3], K. Ibuki[2], T. Miura[2], M. Hayami[2] & H. Takahashi[1,3]

1 Laboratory of Virus Control, Institute for Virus Research, Kyoto University, 53 Shogoin-Kawahara-cho, Kyoto 606-8507, Japan
2 Laboratory of Primate Model, Institute for Virus Research, Kyoto University, 53 Shogoin-Kawahara-cho, Kyoto 606-8507, Japan
3 Department of Microbiology and Immunology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

Key words
African green monkey; CD1d; chimpanzee; HIV-1/ SIV; NKT; primate; rhesus monkey; susceptibility

Correspondence
Hidemi Takahashi, MD, PhD
Department of Microbiology and Immunology
Nippon Medical School
1-1-5, Sendagi, Bunkyo-ku
Tokyo 113-8602, Japan
Tel: 81 3 3822 2131x5381
Fax: 81 3 3316 1904
e-mail: htkuhkai@nms.ac.jp

Received 15 June 2005; revised 15 August 2005; re-revised 28 August 2005; accepted 2 September 2005

doi: 10.1111/j.1399-0039.2005.00504.x

Abstract

The hereditary conservation in the genetically encoded *CD1D* sequences of various primates was analyzed. Genomic *CD1D* sequences of 17 rhesus macaques with distinct origins, eight Indian and nine Chinese, were examined and differences of only one or two nucleotides were detected and the consensus sequence of rhesus *CD1D* was determined. *CD1D* consensus sequences of three African green monkeys (AGMs) and the rhesus monkeys were then compared to study the evolutionary differences among interspecies. The *CD1D* consensus sequence determined from AGMs apparently differed by seven nucleotides from the rhesus consensus sequence, and nucleotide difference induced only three amino acid changes within Exon3, corresponding to the α_2 domain of CD1d having a hydrophobic ligand-binding pocket. Such changes in the α_2 domain may alter the characteristics of the SIV-derived glycolipid/lipid antigens presented by each CD1d molecule to innate natural killer T cells. In addition, the *CD1D* genomic sequences of three chimpanzees (chimps) were determined. To our surprise, although Exon2 and Exon3 reflecting antigen-binding α_1 and α_2 domains in chimps' *CD1D* were identical to that in humans except one amino acid, three amino acids within Exon4, reflecting α_3 domain, were distinct from humans, and one of them was identical to those in rhesus and AGM *CD1D*. On the basis of the findings, the evolutionary relationship of the CD1d molecules among the various primates and their HIV-1/SIV susceptibility will be discussed.

Introduction

Unlike B lymphocytes bearing immunoglobulin receptors that can bind directly onto various free antigens, T lymphocytes can recognize foreign antigens as their processed fragments in conjunction with antigen-presenting molecules on the surface of antigen-presenting cells (APCs) (1). Such antigen-presenting molecules are usually classified into three groups: class I major histocompatibility complex (MHC), class II MHC, and CD1. The first two MHC molecules can present processed peptide antigens, whereas the last CD1 can present glycolipid/lipid antigens derived from various pathogens (2). In general, endogenously synthesized foreign antigens such as viral proteins are degraded into peptide fragments composed of eight to 10 amino acids in the cytosol through an ATP-dependent proteolytic system, trapped into the class I MHC groove and presented to specific $CD8^+$ T lymphocytes (3). In contrast, free soluble and particulate peptide antigens from various pathogens are captured exogenously by APCs such as immature dendritic cells (iDCs), transported to acidic endosomal compartment, where the captured antigens are further degraded to fit into the channel of class II MHC, and the peptide-loaded class II MHC molecules are exported to the cell surface for the stimulation of antigen-specific $CD4^+$ T lymphocytes (4).

As for CD1 antigen presentation, there have been four distinct types of CD1 molecules (CD1a, b, c, and d) identified on the surface of human APCs, and two subclasses

of CD1d are found in mice to present glycolipid/lipid but not conventional processed peptide antigens (5, 6). On the basis of sequence homologies, these CD1 molecules can be classified into two types: CD1a, b, and c as group 1 and CD1d as group 2 (7). These CD1 molecules present unique glycolipid/lipid antigens with amphipathic structure whose hydrophobic lipid tails are bound to deep cavity in the CD1s and present lipid–sugar complex to T cells expressing αβ T-cell receptors (TCRs). For example, CD1a presents dideoxymycobactin (DDM) (8), CD1b lipoarabinomannan (LAM) (9), glucose monomycolate (GMM) (10), and mycolic acid (11); CD1c mannosyl phosphoisoprenoid (12) and CD1d glycosyl-phosphatidyl inositol (GPI) (13); or α-galactosyl ceramide (αGalCer) (14). Such glycolipid/lipid antigens presented by the group 1 CD1 molecule (CD1a, b, and c) on DCs stimulate conventional αβ TCR-bearing T cells expressing either $CD8^+$ T cells or $CD4^-CD8^-$ T cells, whereas antigens presented by group 2 CD1 (CD1d) on DCs, as well as various types of mononuclear cells, such as T cells, B cells, and monocytes, activate natural killer T (NKT) cells. NKT cells are a subset of T cells expressing not only NK receptor but also invariant αβ TCRs such as Vα24 TCR in humans (15) and Vα14 in mice (16) for the recognition of glycolipid/lipid antigens like αGalCer presented by the CD1d.

The structures of these CD1 molecules are similar to those of class I MHCs bearing noncovalently bound β_2 microglobulin; however, the CD1s show limited polymorphism and do not map to the MHC genes (17). Moreover, although class I and II MHC molecules are extremely diverse among species with self-restricted elements that can present processed antigens only to the same MHC-bearing cells, the CD1-encoding genes are highly conserved, and their structures are shared among species (18), indicating the importance of CD1-restricted effectors for maintaining species evolution, and mutation or inactivation of the CD1s might deprive the species continuity. Furthermore, the fact that mice and rats have only CD1d among other classical CD1s in a highly conserved manner (19) reveals that the preservation of CD1d molecules together with NKT cells as their evolutionarily selected partners seems to be critical for such rodents to evolve.

In the present study, we focused on analyzing the hereditary conservation in the genetically encoding *CD1D* sequences of various primates. To date, human and rodent *CD1D* sequences (20–22) and some portion of genomic DNA of rhesus macaque have been reported (23). Therefore, we first confirmed whether the genomic DNA sequence of *CD1D* was identical to the cDNA encoding *CD1D* message by establishing iDCs from peripheral blood mononuclear cells (PBMCs) of rhesus macaques to determine the *CD1D* consensus sequence. We then compared the *CD1D* sequences of African green monkeys (AGMs) and rhesus monkeys to see the evolutionary difference in the CD1d molecules among interspecies. Finally, we determined for the first time the genomic sequences of *CD1D* in chimpanzees (chimps) for comparison. On the basis of the findings obtained here, the evolutionary relationship in the CD1d molecules among various primates and their HIV-1/SIV susceptibility as models for HIV-1 infection will be discussed.

Materials and methods

Animals

Seventeen rhesus macaques (*Macaca mulatta*), nine Chinese originated and eight Indian originated, and three AGMs (*Cercopithecus aetiops grivet*) were used in this experiment. All monkeys used in this study were housed in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University. Three blood samples of chimps (*Pan troglodytes*) were kindly obtained from Dr S. Singh (Cornell University).

Induction of iDCs from PBMCs

PBMCs of rhesus macaques were isolated from heparinized blood by density gradient centrifugation using Lymphocyte Separation Solution (Nakarai Tesque, Kyoto, Japan) and cultured in monocyte-separating plates (MSP-P) (JIMRO, Gunma, Japan) at 1×10^7 cells in 2.5 ml of Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, Grand Island, NY), supplemented with 10% fetal calf serum (FCS) for 1–2 h in a 5% CO_2 incubator at 37°C as described recently (24). After incubation, the non-adherent cells were removed, and the adherent monocytes were further cultured in 2.5 ml DMEM supplemented with 10% FCS, 200 ng/ml recombinant human granulocyte macrophage-colony-stimulating factor (rhGM-CSF) (R&D system, Minneapolis, MN), and 50 ng/ml recombinant human interleukin-4 (rhIL-4) (R&D system) for an additional 6–8 days to generate the CD1-expressing monocyte-derived iDCs as described previously (25).

RNA extraction from rhesus iDCs and cDNA synthesis

RNAs were extracted from the established monocyte-derived iDCs using total RNA isolation reagent for the liquid samples (TRIZOL; Gibco-BRL). Then poly-A-tailed mRNAs were collected from the extracted total RNA and changed into cDNA with a first-strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA).

Cloning of *CD1D*-encoding genes

Preparation of PCR product

In order to proceed with the cloning of rhesus *CD1D* genes, the cDNA obtained was added to a mixture

containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each dNTPs, 0.2 μM of each primer, and 2.5 units of AmpliTaq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The amplification of cDNA fragments was performed for 35 cycles consisting of the following three steps: denaturation at 94°C for 60 s (5 min for the first cycle), annealing at 55°C for 60 s, and elongation at 72°C for 90 s (5 min for the last cycle). The primers shown below were estimated on the basis of human *CD1D* sequences (26): CD1d (forward) (5′-TTCACA GGACGCCCTGATAGGAACTTTGCCTCTTAAACC-GGGAGGTAAAGCCCAC-3′) and CD1d (reverse) (5′-TCAGGACGCCCTGATAGGAACTTTGCCTCTTAA-ACCGGGAGGTAAAGCCCAC-3′).

Subcloning of PCR product

The PCR products were then run in a 1.5% agarose gel supplemented with ethidium bromide. The specified bands were excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified cDNAs were ligated into a pCR2.1-TOPO TA cloning vector (Invitrogen) and inserted into their competent cells (*Escherichia coli*-derived DH5α; Invitrogen). After 24 h incubation, white colonies were picked for sequencing experiments. Twelve white colonies from LB plate were picked up and the sequence analysis was performed on four clones. Three sequencing experiments were carried out to account for false positive.

Sequence analysis of cloned cDNA

Sequence analysis was performed using a DNA sequencing Kit (Big Dye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems, Foster city, CA). The amplification of cDNA fragments was performed for 25 cycles, consisting of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and elongation at 60°C for 4 min. Sequences of amplified DNA fragments were determined with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). M13 primers were used for sequencing analysis: M13 (forward) (5′-GTAAAACGACGGCCAG-3′) and M13 (reverse) (5′-CAGGAAACAGCTATGAC-3′).

Direct sequencing of *CD1D* from genomic DNA

Genomic DNA was extracted from PBMCs of various primates with QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). Then the genomic DNA was subjected to the PCR. The following primers were prepared on the basis of human genomic *CD1D* sequence (26):

Exon1	E1F	(5′-AGAAGAGTGCGCAGGTCAGAGG GCGGCCGC-3′)
	E1R	(5′-CGCCAGTGCGTCCCGGGTCTCAC CAGT CCC-3′)
Exon2	E2F	(5′-ATGCTGGCTGCTCTCCCGGCC ACTTGCTAC-3′)
	E2R	(5′-TCTGTGGCCATTCTCTCCCCTT GGGTAC CG-3′)
Exon3	E3F	(5′-TCTCTTTCATCTCTCCCAGTCT TTTAAAC-3′)
	E3R	(5′-GGAAAAGCCCGTTTGGAGCCC TGAAG TGGA-3′)
Exon4	E4F	(5′-GGAGCCTCTAATGCAGAGTTT TCACTTTAA-3′)
	E4R	(5′-CACCCCAGTGCCTCCCTCTAT GCCTGA GGA-3′)
Exon5–6	E5F	(5′-ATTGGGGTTTTAAGTGGAG GAGGAAATAAG-3′)
	E5R	(5′-TTTCAAGGTATCTCTCCTATCT TCAATT CC-3′)

The amplification of DNA fragments was performed for 35 cycles, consisting of denaturation at 94°C for 30 s (5 min for the first cycle), annealing at 60°C for 30 s, and elongation at 72°C for 45 s (5 min for the last cycle) and confirmed with agarose gel electrophoresis. The DNA fragments were purified and then analyzed with an ABI PRISM 3100 Genetic Analyzer. In the case of the thin fragments, they were ligated into the TA cloning site of pCR 2.1-TOPO Vector (TOPO TA Cloning; Invitrogen) and inserted into their competent cells (*Escherichia coli*-derived DH5α; Gibco-BRL). The plasmid was prepared by the alkaline lysis method (27), and the purified plasmid was used for sequence analysis.

GenBank accession

Each determined sequence of the *CD1D* was submitted to public database and obtained the GenBank accession numbers as follows:

C-1 to C-7 and C-9 are corresponding to the numbers of AB222829 to AB222836, respectively, and C-8 is to AB232047. I-1 to I-8 are corresponding to the numbers of AB222837 to AB222844, respectively. Three AGMs are corresponding to the numbers of AB222994 to AB222996 and three chimps are to AB222997, AB222998, and AB223044, respectively.

Results

Establishment of iDCs from PBMCs of a rhesus macaques and the cloning and sequencing of their CD1d molecules

Because CD1d molecules are generally expressed on DCs or iDCs, we first established the iDCs from freshly isolated PBMCs of a rhesus macaque named MM257 by culturing them with GM-CSF and rhIL-4, as previously indicated. Then, total RNA was extracted from those iDCs, and the cDNA of *CD1D* was cloned from the mRNA using reverse

transcriptase. As shown in Figure 1, the size of rhesus *CD1D* cDNA was 1014 bp, similar to that of human *CD1D* that has been reported previously (26). The cloned cDNA was inserted into competent cells for sequence analysis using a TA-cloning vector. Consequently, we obtained four clones, and their sequences were analyzed using a Genetic Sequencing Analyzer. As represented in Figure 2, we managed to determine the *CD1D* sequence of rhesus MM257. However, since only human GM-CSF and IL-4 were available to induce DCs from various primates, we have experienced serious difficulty in establishing primates' DCs from PBMCs. We thus attempted to confirm whether the genomic DNA sequence for *CD1D* was similar to the *CD1D* message encoded in the cDNA to determine the *CD1D* consensus sequence and found that both sequences were identical in the rhesus MM257 (data not shown). Therefore, we examined the genomic DNA instead of the mRNA-derived cDNA for further analysis.

Comparison of *CD1D* sequences among rhesus macaques with distinct origins

In contrast to class I and class II MHC molecules composed of distinct sequences of amino acids in each individual even among the same species, CD1 molecules consist of species-specific and highly conserved sequences (22). Therefore, we speculated that the *CD1D* sequence of MM257 would probably be the same as other rhesus macaques. We examined the genomic *CD1D* sequences of 16 additional rhesus macaques. The former eight macaques originated from China including MM257 and the latter eight were from India. As expected, in comparison with the whole *CD1D* sequences of MM257, only one or two nucleotide differences could be found among other samples, and the consensus sequence of the rhesus *CD1D* was determined from the results (Table 1). Since these nucleotide changes affected only one amino acid alteration

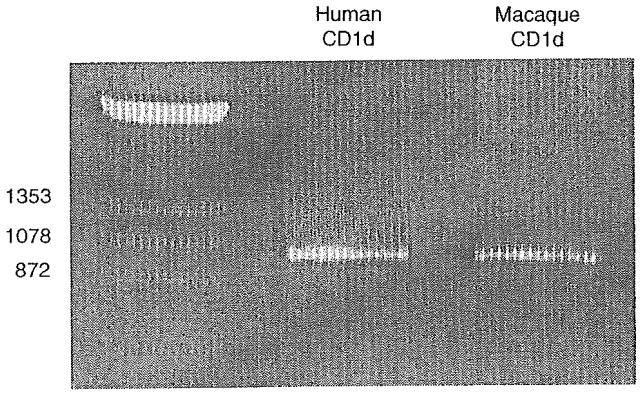


Figure 1 Amplification of the rhesus macaque *CD1D* cDNA from monocyte-derived immature dendritic cells. The size of specific band (bp) obtained by RT-PCR was similar to that of human CD1d.

at position 896, Leu for MM257 and His for other monkeys in Exon5, such changes do not have an influence on antigen presentation by CD1d molecules (28).

Comparison of *CD1D* sequences between rhesus macaques and AGMs

Because the susceptibility to SIV is totally different between rhesus macaques and AGMs in that the rhesus macaques will develop immune-deficient states with marked decrease in CD4, whereas the latter AGMs will not, although AGMs have been thought as natural hosts of SIV (29); we examined whether the *CD1D* sequences were genetically conserved between those species. As shown in Table 2, the consensus *CD1D* sequence determined from the AGMs differed by seven nucleotides from the rhesus consensus sequence. Nevertheless, these nucleotide differences resulted in three amino acid changes within Exon3, corresponding to the α_2 domain of CD1d, having a hydrophobic ligand-binding pocket (28).

Comparison of the consensus *CD1D* sequences of rhesus macaques and AGMs with those of chimps and humans

These rhesus macaques and AGMs are not susceptible to HIV-1, but both chimps and humans are susceptible to HIV-1, but not to SIV (30). We thus conducted direct sequencing for *CD1D* using PBMCs obtained from three chimps (kindly provided by Dr S. Singh, Cornell University) to determine the consensus *CD1D* sequence of chimps. Nucleotide sequences (Figure 3A) and amino acid alignments (Figure 3B) among humans, chimps, rhesus macaques, and AGMs are summarized. Unexpectedly, both Exon2 and Exon3, reflecting the antigen-binding sites of the α_1 and α_2 domains, were identical except for one amino acid, Glu(E), at position 182 between chimps and humans. However, three amino acids, Asp (D), Ala (A), and Ile (I), at positions 229, 252, and 274 within Exon4, reflecting α_3 domain, were distinct from humans, and one of them was identical to those in rhesus and AGM *CD1D*. Moreover, on the basis of recent reports on the precise structural analysis of CD1d molecules for antigen presentation to NKT cells (31–34), we boxed the amino acid sequences crucial for antigen presentation and highlighted the amino acids critical for human NKT-cell activation among them (Figure 3B).

Discussion

In the present study, we first determined the *CD1D* sequence of a rhesus macaque, named MM257, from mRNA-derived cDNA clones and confirmed the consistency of the sequence with genomic DNA obtained from MM257 PBMC to decide the consensus sequence. We then

```
        10         20         30         40         50         60         70         80         90        100        110        120
ATGGGGTGCCTGCTGTTTCTGCTGCTCTGGGCGCTCCTCCAGGCTTGGGGAAGCGCTGAAGTCCCGCAAAGGCTTTTCCCCCTCCGCTGCCTCCAAATCTCGTCCTTCGCCAATAGCAAC
M  G  C  L  L  F  L  L  L  W  A  L  L  Q  A  W  G  S  A  E  V  P  Q  R  L  F  P  L  R  C  L  Q  I  S  S  F  A  N  S  N

       130        140        150        160        170        180        190        200        210        220        230        240
TGGACGCGCACCGATGGTTTGGCGTGGCTGGGGGAGCTGCAGACGCACAGCTGGAGCAACGACTCCGACACCATCCGCTCTCTGAAGCCGTGGTCCCAGGGCACGTTCAGCGACCAGCAG
W  T  R  T  D  G  L  A  W  L  G  E  L  Q  T  H  S  W  S  N  D  S  D  T  I  R  S  L  K  P  W  S  Q  G  T  F  S  D  Q  Q

       250        260        270        280        290        300        310        320        330        340        350        360
TGGGAGGCGCTGCAGCGTGTATTTCGGGTTTATCGAAGCAGCTTCACCAGGGACGTGAAGGAATTCGCCAAAATGCTGCGCTTAGCCTATCCCATGGAGCTCCAGGTGTCTGCTGGCTGT
W  E  A  L  Q  R  V  F  R  V  Y  R  S  S  F  T  R  D  V  K  E  F  A  K  M  L  R  L  A  Y  P  M  E  L  Q  V  S  A  G  C

       370        380        390        400        410        420        430        440        450        460        470        480
GAGGTGCACCCTGGAAACGCCTCACATAACTTCTTCCATGTAGCGTTTCAAGGAAGTGATATCCTGAGTTTCCAAGGAACTTCTTGGGAACCAGCCCAAGAGGCCCCACTTTGGGTAAAC
E  V  H  P  G  N  A  S  H  N  F  F  H  V  A  F  Q  G  S  D  I  L  S  F  Q  G  T  S  W  E  P  A  Q  E  A  P  L  W  V  N

       490        500        510        520        530        540        550        560        570        580        590        600
TTGGCCATCCAAGTGCTCAACCAGGACAACTGGACGAAGGAAACAGTGCAGTGGCTCCTTAATGACACCTGCCCCCAATTTGTCAGTGGCCTCCTTGAGTCAGGGAAGTCGGAACTGGAG
L  A  I  Q  V  L  N  Q  D  N  W  T  K  E  T  V  Q  W  L  L  N  D  T  C  P  Q  F  V  S  G  L  L  E  S  G  K  S  E  L  E

       610        620        630        640        650        660        670        680        690        700        710        720
AAGCAAGTGAAGCCCAAGGCCTGGCTGTCCCGTGGCCCCAGTCCTGGCCCTGGCCGTCTGCAGCTTGTGTGCCATGTCTCAGGATTCTACCCAAAGCCCGTGTGGGTGAAGTGGATGCGG
K  Q  V  K  P  K  A  W  L  S  R  G  P  S  P  G  P  G  R  L  Q  L  V  C  H  V  S  G  F  Y  P  K  P  V  W  V  K  W  M  R

       730        740        750        760        770        780        790        800        810        820        830        840
GGTGAGCAGGAGCAGCAGGGCACTCAGCGAGGGGACATCCTGCCCAATGCTGACGAGACATGGTATCTCCGAGCAACCCTGGAGGTGGCCGCTGGGGAGGCAGCTGGCCTGTCCTGTCGG
G  E  Q  E  Q  Q  G  T  Q  R  G  D  I  L  P  N  A  D  E  T  W  Y  L  R  A  T  L  E  V  A  A  G  E  A  A  G  L  S  C  R

       850        860        870        880        890        900        910        920        930        940        950        960
GTGAAGCACAGCAGTCTAGAGGGCCAGGACATCGTCCTCTACTGGGGTGGGAGCCTCACCTCCGTGGGCTTGATCGCCTTGGCAGTCCTGGCATGCTTGCTGTTCCTCCTTGCACTCATT
V  K  H  S  S  L  E  G  Q  D  I  V  L  Y  W  G  G  S  L  T  S  V  G  L  I  A  L  A  V  L  A  C  L  L  F  L  L  A  L  I

       970        980        990       1000       1010       1020
GTAGGCTTTACCTTCCGGTTTAAGAGGCAAACTTCCTATCAGGGCATCCTGTGA
V  G  F  T  F  R  F  K  R  Q  T  S  Y  Q  G  I  L  *
```

Figure 2 Nucleotide and amino acid sequences of the CD1d molecule for rhesus MM257. PCR product obtained from cDNA of MM257 was inserted into a TA cloning vector and sequenced using a DNA sequencer. Analysis of the sequences was performed by GENETYX-MAC software.

Table 1 Comparison of the *CD1D* nucleotide sequences in rhesus macaques of distinct origins

Chinese											
Position (bp)	Genomic region	C-1[a]	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	Consensus
96	Exon2	A		G				A/G		A/G	A
199		T	C								T
450	Exon3	A		A/G	A/G	A/G	G	A/G	A/G	G	A/G
510		C			G					C/G	C
555		C			C/T					C/T	C
896	Exon5	T	A	A	A	A	A	A	A	A	A
Indian											
Position (bp)	Genomic region	I-1	I-2	I-3	I-4	I-5	I-6	I-7	I-8	Consensus	
96	Exon2	A				G	A/G	A/G	G	A	
199		T	A/T		C/T					T	
331	Exon3	C		C/T		C/T	C/T		T	C	
443		C	A/C		A/C					C	
450		A/G		G	G		G		G	A/G	
510		C			C/G		A/G	C/G		C	
555		C			C/T		C/T	C/T		C	
659	Exon4	T			G					T	
838		C			C/T		C/T	C/T		C	

Genomic *CD1D* sequences of 17 rhesus macaques, nine Chinese (C-1 to C-9), and eight Indian (I-1 to I-8) origins were examined, and the consensus sequence of the *CD1D* for rhesus macaques was determined.

[a] C-1 corresponds to MM257 in the text.

Table 2 Comparison of the consensus *CD1D* sequences between rhesus macaques and African green monkeys (AGMs)

		Nucleotide		Amino acid	
Position (bp)	Genomic region	AGM consensus	Macaque consensus	AGM consensus	Macaque consensus
93	Exon2	T	C	L	L
96		G	A	Q	Q
210		T	G	P	P
351	Exon3	C	T	S	S
417		A	T	**R**	**S**
510		G	C	**K**	**N**
518		G	A	**R**	**K**

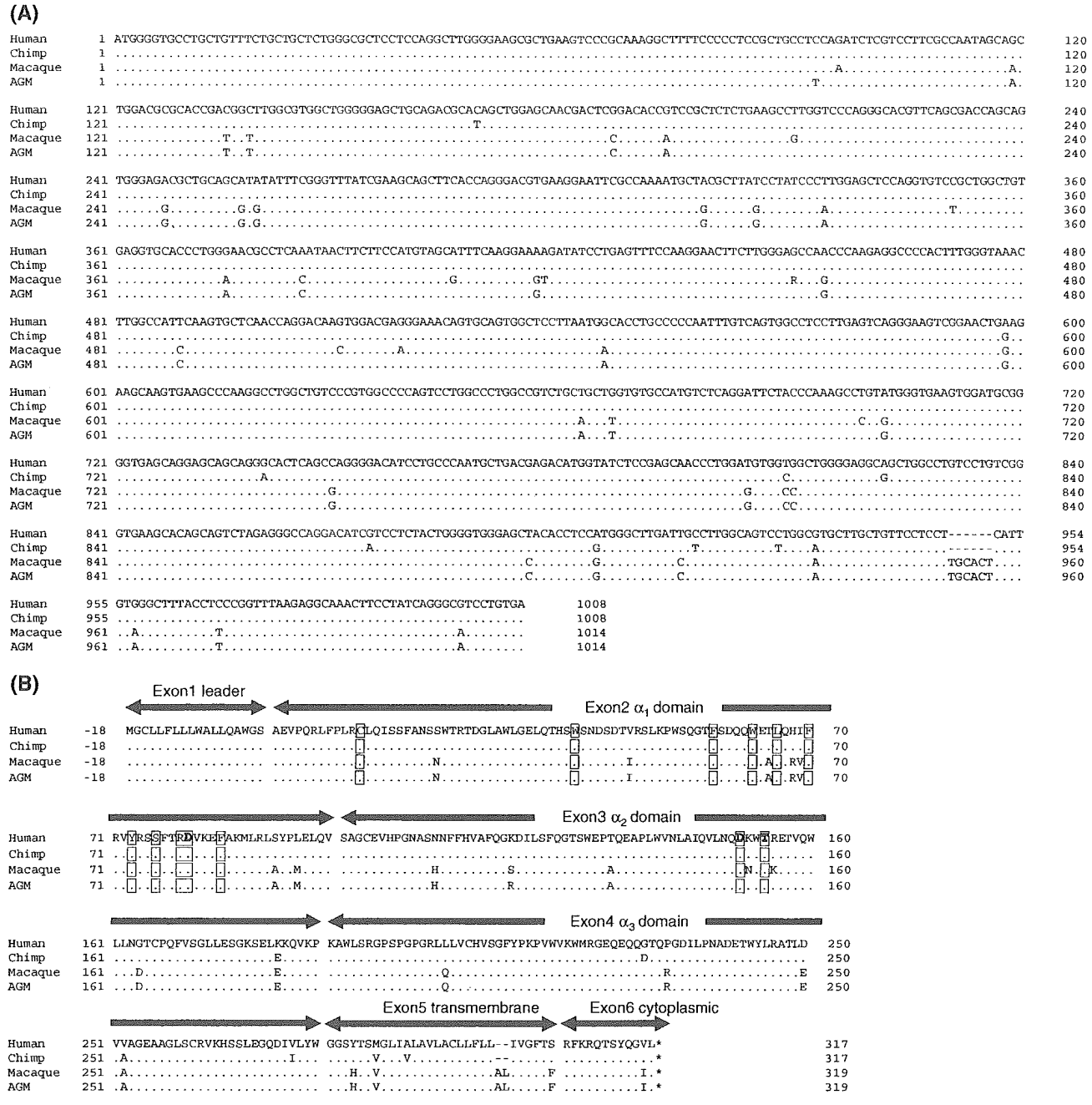
Consensus sequence of the *CD1D* for AGM was determined from three monkeys. Distinct positions for each specific nucleotide and amino acid were shown. Although the consensus *CD1D* sequence of AGMs differed by seven nucleotides from the rhesus consensus sequence, these nucleotide differences induced only three amino acid changes within Exon3 (indicated as bold).

examined the genomic DNA of another 16 macaques with distinct origins and found that the *CD1D* sequence of all the monkeys tested was identical except one amino acid in Exon5. Thus, genetic structure of species-specific CD1d molecules was far more tightly conserved than that expected among the rhesus macaques and would not be affected by long-term environmental stimulation. Also, the results suggest that the mutation of the *CD1D* genes might ruin the species and indicate the importance of the CD1d-restricted NKT-related immunity for species survival. Indeed, we have recently found that the syngeneic thymoma cells were deleted by NKT-like cells when they expressed unfavorable encoding genes such as alpha-fetoprotein (35) and that dysfunction of NKT cells causes autoimmune disorders (36) or deficiencies of CD1d seems to be associated with the impairment of various infectious diseases (37) that might correlate with the disappearance of the primates bearing mutated *CD1D*.

We next examined whether the *CD1D* sequences were genetically conserved between the two distinct types of monkeys, rhesus macaques and AGMs, and found that the *CD1D* consensus sequence determined from the AGMs differed by seven nucleotides from the consensus sequence of rhesus macaques, and those nucleotide differences generated three amino acids changes within Exon3. Because Exon3 corresponds to the α_2 domain of the CD1d molecules having a hydrophobic ligand-binding pocket critical for antigen presentation (28), these changes of the amino acids may alter the capacity of the presented glycolipid/lipid antigens to effector NKT cells. In the case of SIV infection, such changes in the α_2 domain may affect the characteristics of the SIV-derived glycolipid/lipid antigens presented by the CD1d and may induce functional differences of the activated NKT cells that may result in the distinct susceptibility of the monkeys to SIV. If the different susceptibilities to SIV (38) correlate with the fact that AGMs are the coexisting natural hosts of SIV and rhesus macaques are the victims, NKT-associated innate immunity may have some relation to those opposite outcomes. Also, the finding that the amino acid sequences of Exon2 and Exon4 corresponding to α_1 and α_3 domains of the CD1d, respectively, were totally identical between the rhesus and AGMs indicates that the domains would be conserved among monkeys, and changes of the amino acid sequences at the sites would destroy the species continuation.

It is widely known that genomic sequences between humans and chimps are highly conserved, and both are susceptible to HIV-1 but very weakly to SIV as reported previously that SIVcpz has been isolated from chimpanzee (39). Thus, we examined the *CD1D* sequence of chimps and determined their consensus sequence for the first time. Interestingly, both α_1 and α_2 domains of the CD1d of chimps were totally identical to humans except for one amino acid at the end of the Exon3; however, three amino acids in Exon4 were distinct from humans, and one of them was the same as identified in the α_3 domain of rhesus macaques and AGMs. In comparison with class I MHC molecules, such α_3 domain of CD1 has been reported to be firmly preserved among each species (20). Moreover, as shown in the Figure 3B, all the crucial amino acids for lipid/glycolipid antigen presentation were strictly conserved among the primates tested, and thus the presented antigens by the α_1 and α_2 domains of CD1d molecule must be the similar among those primates. These results suggest that NKT response to HIV or SIV may not be determined by the antigens presented by the species-specific CD1d molecules, but rather by the structural difference in the CD1d itself or antigen/CD1d complex. Indeed, as shown in Figure 3B, highlighted amino acids at positions Asp(D)151 and Thr(T)154, critical for human NKT-cell activation to the known glycolipid antigens αGalCer (34), were associated with Lys(K)152 and Arg(R)155 in the α_2 domain of the AGM, human, and chimps' CD1d, while they were linked with Asn(N)152 and Lys(K)155 of macaque CD1d, suggesting that distinct susceptibility to SIV may correlate with the CD1d structure and with the activated status of NKT cells because only rhesus macaques fall into immune-deficient state via SIV infection among them.

Taken together, the findings shown in the present study reveal that the α_1 and α_2 domains of the CD1d molecules may present retroviral glycolipid/lipid antigens to the species-specific NKT cells, which may be associated with the type of infectious viruses as well as the susceptibility to them and suggest the evolutionary relationship between the species-specific CD1d molecules and retrovirus infection through the activation of innate effector NKT cells.



Figures 3 Comparison of consensus *CD1D* sequences between chimpanzees (chimps) and monkeys on the basis of human consensus sequence. (A) Nucleotides alignment of CD1d molecules for each subject. The first line stands for the known human *CD1D*, the second line for the determined chimps' *CD1D*, third line for the rhesus macaques, and the last line for the African green monkeys (AGMs). When the nucleotide was the same with humans, the positions were indicated with dot. (B) Amino acid alignment of CD1d molecules for each subject. Antigen-binding sites, Exon2 and Exon3, were indicated with arrows. Also, the amino acid sequences crucial for antigen presentation were boxed, and those critical for human natural killer T-cell activation among them were highlighted.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Science, Sport, and Culture, from the Ministry of Health and Labor and Welfare, from the Japanese Health Sciences Foundation, and from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

References

1. Takahashi H. Antigen processing and presentation. *Microbiol Immunol* 1993: **37**: 1–9.
2. Porcelli SA, Segelke BW, Sugita M, Wilson IA, Brenner MB. The CD1 family of lipid antigen-presenting molecules. *Immunol Today* 1998: **19**: 362–8.
3. Takahashi H. Antigen presentation in vaccine development. *Comp Immunol Microbiol Infect Dis* 2003: **26**: 309–28.
4. Nijman HW, Kleijmeer MJ, Ossevoort MA et al. Antigen capture and major histocompatibility class II compartments of freshly isolated and cultured human blood dendritic cells. *J Exp Med* 1995: **182**: 163–74.
5. Burdin N, Kronenberg M. CD1-mediated immune responses to glycolipids. *Curr Opin Immunol* 1999: **11**: 326–31.
6. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol* 2004: **22**: 817–90.
7. Gumperz JE, Brenner MB. CD1-specific T cells in microbial immunity. *Curr Opin Immunol* 2001: **13**: 471–8.
8. Moody DB, Young DC, Cheng TY et al. T cell activation by lipopeptide antigens. *Science* 2004: **303**: 527–31.
9. Sieling PA, Chatterjee D, Porcelli SA et al. CD1-restricted T cell recognition of microbial lipoglycan antigens. *Science* 1995: **269**: 227–30.
10. Moody DB, Reinhold BB, Guy MR et al. Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. *Science* 1997: **278**: 283–6.
11. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. Recognition of a lipid antigen by CD1-restricted alpha beta$^+$ T cells. *Nature* 1994: **372**: 691–4.
12. Moody DB, Ulrichs T, Muhlecker W et al. CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. *Nature* 2000: **404**: 884–8.
13. Joyce S, Woods AS, Yewdell JW et al. Natural ligand of mouse CD1d1: cellular glycosylphosphatidylinositol. *Science* 1998: **279**: 1541–4.
14. Kawano T, Cui J, Koezuka Y et al. CD1d-restricted and TCR-mediated activation of Valpha14 NKT cells by glycosylceramides. *Science* 1997: **278**: 1626–9.
15. Exley M, Garcia J, Balk SP, Porcelli S. Requirements for CD1d recognition by human invariant Valpha24$^+$ CD4$^-$CD8$^-$ T cells. *J Exp Med* 1997: **186**: 109–20.
16. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1$^+$ T lymphocytes. *Science* 1995: **268**: 863–5.
17. Jayawardena-Wolf J, Bendelac A. CD1 and lipid antigens: intracellular pathways for antigen presentation. *Curr Opin Immunol* 2001: **13**: 109–13.
18. Couedel C, Peyrat MA, Brossay L et al. Diverse CD1d-restricted reactivity patterns of human T cells bearing 'invariant' AV24BV11 TCR. *Eur J Immunol* 1998: **28**: 4391–7.
19. Matsuura A, Takayama S, Kinebuchi M et al. RT1.P, rat class Ib genes related to mouse TL: evidence that CD1 molecules but not authentic TL antigens are expressed by rat thymus. *Immunogenetics* 1997: **46**: 293–306.
20. Balk SP, Bleicher PA, Terhorst C. Isolation and characterization of a cDNA and gene coding for a fourth CD1 molecule. *Proc Natl Acad Sci USA* 1989: **86**: 252–6.
21. Balk SP, Bleicher PA, Terhorst C. Isolation and expression of cDNA encoding the murine homologues of CD1. *J Immunol* 1991: **146**: 768–74.
22. Katabami S, Matsuura A, Chen HZ, Imai K, Kikuchi K. Structural organization of rat CD1 typifies evolutionarily conserved CD1D class genes. *Immunogenetics* 1998: **48**: 22–31.
23. Kashiwase K, Kikuchi A, Ando Y et al. The CD1d natural killer T-cell antigen presentation pathway is highly conserved between humans and rhesus macaques. *Immunogenetics* 2003: **54**: 776–81.
24. Shinya E, Owaki A, Shimizu M et al. Endogenously expressed HIV-1 nef down-regulates antigen-presenting molecules, not only class I MHC but also CD1a, in immature dendritic cells. *Virology* 2004: **326**: 79–89.
25. Ichikawa M, Sugita M, Takahashi M et al. Breast milk macrophages spontaneously produce granulocyte-macrophage colony-stimulating factor and differentiate into dendritic cells in the presence of exogenous interleukin-4 alone. *Immunology* 2003: **108**: 189–95.
26. Calabi F, Jarvis JM, Martin L, Milstein C. Two classes of CD1 genes. *Eur J Immunol* 1989: **19**: 285–92.
27. Stadler J, Lemmens R, Nyhammar T. Plasmid DNA purification. *J Gene Med* 2004: **6**: S54–66.
28. Zeng Z, Castano AR, Segelke BW, Stura EA, Peterson PA, Wilson IA. Crystal structure of mouse CD1: an MHC-like fold with a large hydrophobic binding groove. *Science* 1997: **277**: 339–45.
29. Beer BE, Bailes E, Goeken R et al. Simian immunodeficiency virus (SIV) from sun-tailed monkeys (*Cercopithecus solatus*): evidence for host-dependent evolution of SIV within the *C. lhoesti* superspecies. *J Virol* 1999: **73**: 7734–44.
30. Puissant B, Abbal M, Blancher A. Polymorphism of human and primate RANTES, CX3CR1, CCR2 and CXCR4 genes with regard to HIV/SIV infection. *Immunogenetics* 2003: **55**: 275–83.
31. Zajonc DM, Cantu C III, Mattner J et al. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat Immunol* 2005: **6**: 810–8.
32. Moody DB, Zajonc DM, Wilson IA. Anatomy of CD1-lipid antigen complexes. *Nat Rev Immunol* 2005: **5**: 387–99.
33. Giabbai B, Sidobre S, Crispin MD et al. Crystal structure of mouse CD1d bound to the self ligand phosphatidylcholine: a molecular basis for NKT cell activation. *J Immunol* 2005: **175**: 977–84.
34. Koch M, Stronge VS, Shepherd D et al. The crystal structure of human CD1d with and without alpha-galactosylceramide. *Nat Immunol* 2005: **6**: 819–26.
35. Kim Y, Nakagawa Y, Sugiyama H et al. Induction of CD4$^+$ murine natural killer T-like cells by immunization with syngeneic thymoma expressing embryonic alpha-fetoprotein. *Cell Immunol* 2003: **226**: 1–10.

36. Kojo S, Adachi Y, Keino H, Taniguchi M, Sumida T. Dysfunction of T cell receptor AV24AJ18$^+$, BV11$^+$ double-negative regulatory natural killer T cells in autoimmune diseases. *Arthritis Rheum* 2001: **44**: 1127–38.
37. Hansen DS, Schofield L. Regulation of immunity and pathogenesis in infectious diseases by CD1d-restricted NKT cells. *Int J Parasitol* 2004: **34**: 15–25.
38. Cranage MP, Cook N, Stott EJ, Cook R, Baskerville A, Greenaway PJ. Transmission studies with simian immunodeficiency virus of macaques; persistent infection of baboons. *Intervirology* 1992: **34**: 53–61.
39. Huet T, Cheynier R, Meyerhans A, Roelants G, Wain-Hobson S. Genetic organization of a chimpanzee lentivirus related to HIV-1. *Nature* 1990: **345**: 356–9.

Microbiol. Immunol., 49(7), 667–679, 2005

Editor-Communicated Paper

Intrathymic Effect of Acute Pathogenic SHIV Infection on T-Lineage Cells in Newborn Macaques

Hajime Suzuki[1], Makiko Motohara[1], Ariko Miyake[1], Kentaro Ibuki[1], Yoshinori Fukazawa[1], Katsuhisa Inaba[1], Kyoko Masuda[2], Nagahiro Minato[2], Hiroshi Kawamoto[3], Masanori Hayami[1], and Tomoyuki Miura*,[1]

[1]*Laboratory of Primate Model, Institute for Virus Research, Kyoto University, Kyoto, Kyoto 606–8507, Japan,* [2]*Graduate School of Biostudies, Kyoto University, Kyoto, Kyoto 606–8501, Japan, and* [3]*RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa 230–0045, Japan*

Communicated by Dr. Hidechika Okada: Received April 22, 2005. Accepted April 25, 2005

Abstract: **We intrarectally infected newborn macaques with a pathogenic simian/human immunodeficiency virus (SHIV) that induced rapid and profound CD4$^+$ T cell depletion, and examined the early effects of this SHIV on the thymus. After intrarectal infection, viral loads were much higher in the thymus than in other lymphoid tissues in newborns. In contrast, no clear difference was seen in the viral loads of different tissues in adults. Histological and immunohistochemical observations showed severe thymic involution. Depletion of CD4$^+$ thymocytes began in the medulla at 2 weeks post infection and spread over the whole thymus. After *in vivo* infection, the CD2$^+$ subpopulation, which represents a relatively later stage of T cell progenitors, was selectively reduced and development of thymocytes from CD3$^-$CD4$^-$CD8$^-$ cells to CD4$^+$CD8$^+$ cells was impaired. These results suggest that profound and irreversible loss of CD4$^+$ cells that are observed in the peripheral blood of SHIV-infected monkeys are due to destruction of the thymus and impaired thymopoiesis as a result of SHIV infection in the thymus.**

Key words: **SHIV, Newborn, Thymopoiesis, Rhesus monkey**

The thymus is the primary organ of thymopoiesis and is highly active during early life. Clinical and experimental evidence suggests that the thymus is one of the important target organs for human immunodeficiency virus type 1 (HIV-1) infection (14, 37, 44). Histological studies of the thymus in HIV-1 infected children have revealed an association between abnormal morphological changes including thymic involution, thymocyte depletion and disruption of microenvironments and rapid progression of disease (3, 22, 31, 34). Although the initial interaction between virus and host is considered to be critical in the pathogenesis of HIV-1, detailed analysis of HIV-infected humans at the early phase of infection are extremely limited and nearly impossible to conduct serially (30, 40). Therefore, detailed studies of the thymus using animal models are needed to understand the pathogenicity of HIV.

HIV pathogenesis in the thymus has been investigated in several experimental models. In particular, thymic infection and destruction have been mainly investigated using simian immunodeficiency virus (SIV)/macaque and simian/human immunodeficiency virus (SHIV)/macaque models (16, 17, 26, 27, 41, 42, 45, 48) because the physical structure of monkeys and symptoms of induced immunodeficiency in monkeys are close to those of human. A great advantage of the SHIV/macaque model is that the SHIV contains the HIV *env* gene, which determines virus coreceptor usage

**Address correspondence to* Dr. Tomoyuki Miura, Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, 53 Shogoinkawara-machi, Sakyo-ku, Kyoto, Kyoto 606–8507, Japan. Fax: +81–75–761–9335. E-mail: tmiura@virus.kyoto-u.ac.jp

Abbreviations: AIDS, acquired immunodeficiency syndrome; CD4SP, CD3$^+$CD4$^+$CD8$^-$ single positive; CD8SP, CD3$^+$CD4$^-$CD8$^+$ single positive; DP, CD4$^+$CD8$^+$ double positive; dpi, days post-inoculation; FTOC, fetal thymus organ culture; HIV, human immunodeficiency virus; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; R5, CCR5; SHIV, simian/human immunodeficiency virus; SIV, simian immunodeficiency virus; $TCID_{50}$, 50% tissue culture infectious dose; TdT, terminal deoxynucleotidyl transferase; TN, CD3$^-$CD4$^-$CD8$^-$ triple negative; X4, CXCR4.

and cell tropism (19), and is a critical factor controlling replication in thymus (50).

Several SHIVs have been constructed and examined for infectivity in macaques (20, 28, 41, 47). After *in vivo* passage or anti-CD8 treatment, some of these viruses acquired acute pathogenicity, inducing profound $CD4^+$ T cell depletion and thymic atrophy in the early phase of infection (15, 21, 41, 49). Although this unusually rapid clinical course is not the same as that observed generally in HIV-1 infected humans, the symptoms of SHIV in monkeys have a number of similarities to the symptoms of HIV in humans. Acute pathogenic SHIVs that cause rapid and irreversible $CD4^+$ cell depletion in peripheral blood use CXCR4 (X4) only or in addition to CCR5 (R5) as its coreceptor for entry into susceptible cells (55). In HIV infection, X4-utilization is sufficient to trigger $CD4^+$ cell depletion in human tonsil tissue (38) and the emergence of X4-variants in HIV-infected patients is associated with accelerated progression to acquired immunodeficiency syndrome (AIDS) (4). Taken together, these results indicate that macaques infected with acute pathogenic SHIVs can be used as a model of individuals with emerging X4-utilizing HIV-1, which results in a rapid clinical course.

Although some investigators reported that thymic function might be impaired in HIV-1 infection (6, 12, 43), the effects of infection on the thymus have been controversial. The controversy could probably be resolved if an appropriate animal model and a method for directly measuring thymopoiesis could be found. Recent studies showed that in macaques inoculated with X4-utilizing viruses, massive depletion of peripheral naïve $CD4^+$ T cells was observed within 4 weeks of infection (36, 39). A critical role of the thymus is to supply naïve T cells to the peripheral blood during early life. Thus, a model using a combination of X4-utilizing SHIV and newborn macaques can be useful for clarifying the mechanism of the depletion of peripheral naïve $CD4^+$ T cells associated with thymic dysfunction.

We observed severe impairment of precursor function of $CD3^-CD4^-CD8^-$ triple negative (TN) cells at the early stage of infection with acute pathogenic SHIV in adult monkeys (unpublished data). However, because of thymic atrophy in adults, the morphological and functional changes that occur in the adult thymus as a result of viral infection are generally less informative than the changes that occur in the infant thymus (7). Therefore, to evaluate the effects of acute pathogenic SHIV infection on thymic function, we used newborn macaques to rule out the effect of age-related thymic atrophy. We intrarectally infected two newborns and sacrificed them at 13 days post-infection (dpi) and 26 dpi to carry out virological, biochemical and histopathological analyses. In addition, we examined the effects of aging and viral infection on T cell maturation, employing a fetal thymus organ culture (FTOC) system which is used to culture and develop rhesus monkey thymus progenitor cells in fetal mouse thymus lobe (35). The goals of the present study were to investigate the intrathymic effects of acute pathogenic SHIV (SHIV-C2/1) on T-lineage cells early after mucosal infection and to determine whether thymic dysfunction is related to the rapid clinical course observed in pediatric AIDS. We obtained two interesting results: (1) Virus loads were much higher in the thymus than in other lymphoid tissues in newborn macaques, but not in adults, and (2) thymopoiesis of TN cells was impaired at the early stage of infection.

Materials and Methods

Virus. KS661 is a molecular clone (GenBank ACCESSION No. AF217181) having a consensus sequence of the acute pathogenic SHIV-C2/1 strain, which was generated by *in vivo* passage of SHIV-89.6 (containing *env*, *tat*, *rev* and *vpu* derived from primary isolates of HIV-1 89.6 strain). SHIV-C2/1 was reported to infect macaque monkeys by intrarectal routes and to cause precipitous viremia and drastic depletion of $CD4^+$ cells (49). The virus stock was prepared from the supernatant of a human lymphoid cell line, CEMx174, and stored in liquid nitrogen until use. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus stock was measured in CEMx174. Twenty $TCID_{50}$ was equivalent to one 50% macaque infectious dose (unpublished data).

Animal experiments. Thirteen rhesus macaque monkeys (*Macaca mulatta*) were used in this study. The monkeys included four newborns between 4 and 8 weeks of age at the time of sacrifice, four 3-year-old young macaques, and five adults between 6 and 8 years of age. The monkeys were housed in accordance with regulations approved by the Committee for Experimental Use and Care of Nonhuman Primate in the Institute for Virus Research, Kyoto University. Nine of the 13 monkeys (two of the newborns, all four of the young macaques and three of the adults) were inoculated intrarectally with 2×10^3 $TCID_{50}$ of SHIV-C2/1 KS661 after being anesthetized by intramuscular injection of ketamine chloride. All intrarectal inoculations were done with a pediatric feeding catheter to avoid causing trauma. The catheters were carefully inserted 5 cm into the anus of newborn monkeys and 10 cm into the anus of young and adult monkeys. These nine monkeys were euthanized as follows: One newborn was euthanized at each of 13 and 26 dpi. Two of the young