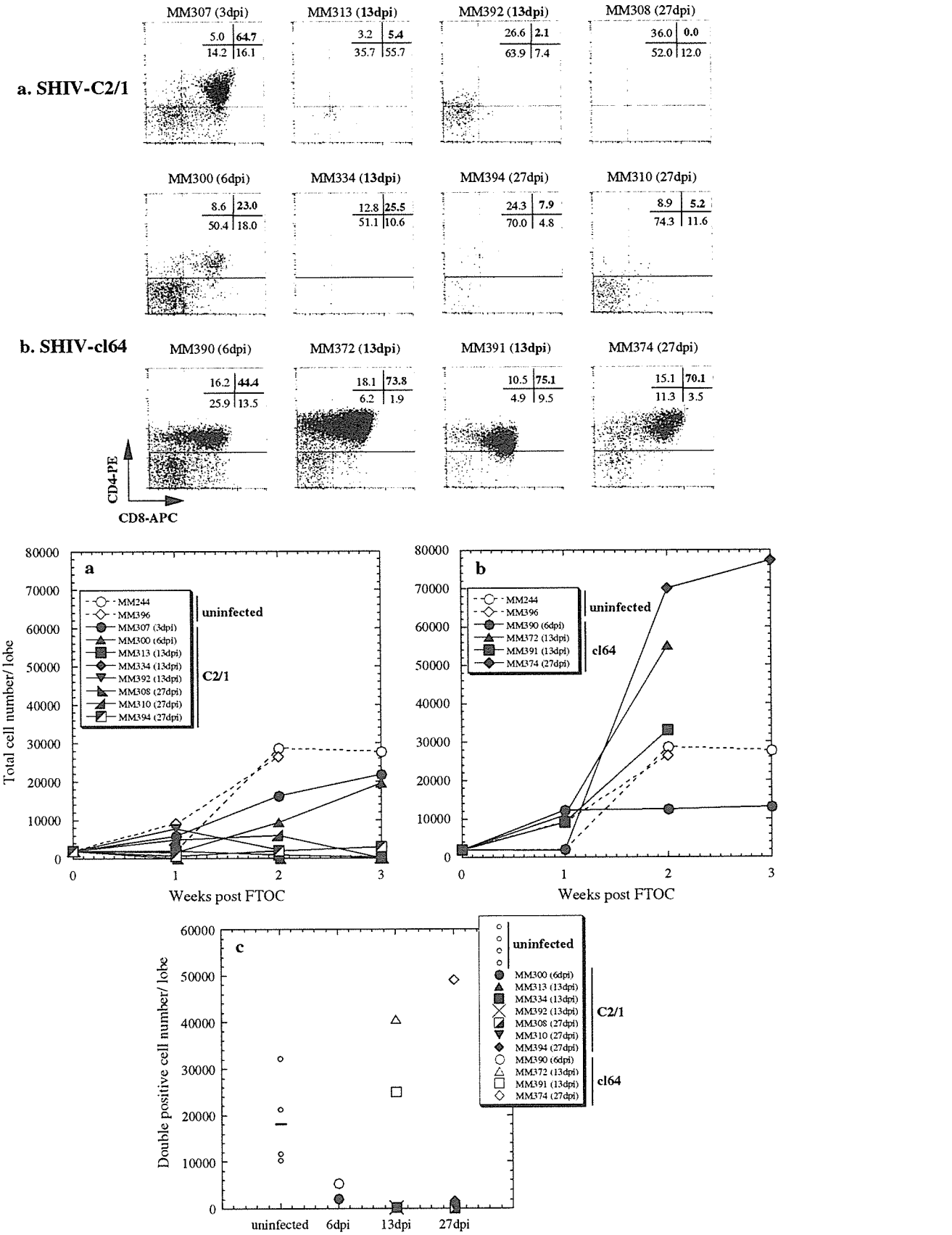

Fig. 5. (A) Comparison of differentiation of CD3 CD4 CD8 TN thymocytes into $CD4^+CD8^+$ DP cells in SHIV-C2/1-infected monkeys (a) and cl64-infected monkeys (b) at 14 days after FTOC. (B) Absolute numbers of living cells per lobe calculated at different time points (1, 2 and 3 weeks after FTOC) in SHIV-C2/1-infected monkeys (a) and cl64-infected monkeys (b). Values are the average numbers of living cells in three lobes. (C) Summary of the competence of CD3 CD4 CD8 TN thymocytes from uninfected, SHIV-C2/1-infected (closed symbols) and SHIV-cl64-infected (open symbols) monkeys to differentiate to $CD4^+CD8^+$ DP cells.

similar in the two types of infection, but the impairment of thymopoiesis was quite different. The rapid decrease of CD4 cells in the very early stage appears to be mainly due to direct killing by the virus infection. Therefore, impaired thymopoiesis adding to the direct killing of virus infection is important for the progressing to profound and irreversible circulating CD4 depletion after 4 weeks, causing immune collapse observed in SHIV-C2/1-infected monkeys.

In conclusion, our results indicate that the acute pathogenic SHIV impairs T-cell differentiation, leading to a profound and irreversible $CD4^+$ T-cell depletion in vivo, and suggest that the thymopoietic potential of the TN thymocytes is predictive of the prognosis after the virus infection. Restoring immune function to intrathymic T-cell progenitors may be a way to treat a multitude of immune disorders that occur in AIDS.

Acknowledgements

We are grateful to James Raymond for proofreading this manuscript, to Yoko Isamoto, Kyoko Yokoyama, Becton Dickinson and DAKO Corp. for technical support. This work was supported by the Cooperation Research of Primate Research Institute, Kyoto University, a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan, a Grant-in-Aid for Scientific Research from the Ministry of Education and Science, Japan and a Research Grant on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation. M.M. is supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology.

References

[1] D. Douek. Thymic output and HIV infection: on the right TREC. Immunity 21 (2004) 744–745.
[2] H. Kaneshima, L. Su, M.L. Bonyhadi, R.I. Connor, D.D. Ho, J.M. McCune. Rapid-high, syncytium-inducing isolates of human immunodeficiency virus type 1 induce cytopathicity in the human thymus of the SCID-hu mouse. J. Virol. 68 (1994) 8188–8192.
[3] M. Rosenzweig, D.P. Clark, G.N. Gaulton. Selective thymocyte depletion in neonatal HIV-1 thymic infection. AIDS 7 (1993) 1601–1605.
[4] V.V. Joshi, J.M. Oleske, A.B. Minnefor, R. Singh, T. Bokhari, R.H. Rapkin. Pathology of suspected acquired immune deficiency syndrome in children: a study of eight cases. Pediatr. Pathol. 2 (1984) 71–87.
[5] D.C. Douek, M.R. Betts, B.J. Hill, S.J. Little, R. Lempicki, J.A. Metcalf, J. Casazza, C. Yoder, J.W. Adelsberger, R.A. Stevens, M.W. Baseler, P. Keiser, D.D. Richman, R.T. Davey, R.A. Koup. Evidence for increased T cell turnover and decreased thymic output in HIV infection. J. Immunol. 167 (2001) 6663–6668.
[6] D.L. Sodora, D.C. Douek, G. Silvestri, L. Montgomery, M. Rosenzweig, T. Igarashi, B. Bernacky, R.P. Johnson, M.B. Feinberg, M.A. Martin, R.A. Koup. Quantification of thymic function by measuring T cell receptor excision circles within peripheral blood and lymphoid tissues in monkeys. Eur. J. Immunol. 30 (2000) 1145–1153.
[7] D.L. Sodora, J.M. Milush, F. Ware, A. Wozniakowski, L. Montgomery, H.M. McClure, A.A. Lackner, M. Marthas, V. Hirsch, R.P. Johnson, D.C. Douek, R.A. Koup. Decreased levels of recent thymic emigrants in peripheral blood of simian immunodeficiency virus-infected macaques correlate with alterations within the thymus. J. Virol. 76 (2002) 9981–9990.
[8] R.A. Weiss, P.R. Clapham, J.N. Weber, A.G. Dalgleish, L.A. Lasky, P.W. Berman. Variable and conserved neutralization antigens of human immunodeficiency virus. Nature 324 (1986) 572–575.
[9] R. Shibata, M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, A. Adachi. Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J. Virol. 65 (1991) 3514–3520.
[10] K.A. Reimann, J.T. Li, R. Veazey, M. Halloran, I.W. Park, G.B. Karlsson, J. Sodroski, N.L. Letvin. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. J. Virol. 70 (1996) 6922–6928.
[11] J.M. Harouse, A. Gettie, R.C. Tan, J. Blanchard, C. Cheng-Mayer. Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. Science 284 (1999) 816–819.
[12] K. Shinohara, K. Sakai, S. Ando, Y. Ami, N. Yoshino, E. Takahashi, K. Someya, Y. Suzaki, T. Nakasone, Y. Sasaki, M. Kaizu, Y. Lu, M. Honda. A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J. Gen. Virol. 80 (Pt 5) (1999) 1231–1240.
[13] R.A. Reyes, D.R. Canfield, U. Esser, L.A. Adamson, C.R. Brown, C. Cheng-Mayer, M.B. Gardner, J.M. Harouse, P.A. Luciw. Induction of simian AIDS in infant rhesus macaques infected with CCR5- or CXCR4-utilizing simian–human immunodeficiency viruses is associated with distinct lesions of the thymus. J. Virol. 78 (2004) 2121–2130.
[14] S.H. Ho, L. Shek, A. Gettie, J. Blanchard, C. Cheng-Mayer. V3 loop-determined coreceptor preference dictates the dynamics of CD4+-T-cell loss in simian–human immunodeficiency virus-infected macaques. J. Virol. 79 (2005) 12296–12303.
[15] J.M. Brenchley, T.W. Schacker, L.E. Ruff, D.A. Price, J.H. Taylor, G.J. Beilman, P.L. Nguyen, A. Khoruts, M. Larson, A.T. Haase, D.C. Douek. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J. Exp. Med. 200 (2004) 749–759.
[16] S. Mehandru, M.A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, M. Markowitz. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J. Exp. Med. 200 (2004) 761–770.
[17] E.A. Berger, P.M. Murphy, J.M. Farber. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. 17 (1999) 657–700.
[18] I.L. Kozyrev, K. Ibuki, T. Shimada, T. Kuwata, T. Takemura, M. Hayami, T. Miura. Characterization of less pathogenic infectious molecular clones derived from acute-pathogenic SHIV-89.6p stock virus. Virology 282 (2001) 6–13.
[19] H. Kawamoto, K. Ohmura, N. Hattori, Y. Katsura. Hemopoietic progenitors in the murine fetal liver capable of rapidly generating T cells. J. Immunol. 158 (1997) 3118–3124.
[20] K. Neben, M. Heidbreder, J. Muller, A. Marxer, H. Petry, A. Didier, A. Schimpl, T. Hunig, T. Kerkau. Impaired thymopoietic potential of immature CD3( )CD4(+)CD8( ) T cell precursors from SIV-infected rhesus monkeys. Int. Immunol. 11 (1999) 1509–1518.
[21] T. Kuwata, T. Igarashi, E. Ido, M. Jin, A. Mizuno, J. Chen, M. Hayami. Construction of human immunodeficiency virus 1/simian immunodeficiency virus strain mac chimeric viruses having vpr and/or nef of different parental origins and their in vitro and in vivo replication. J. Gen. Virol. 76 (Pt 9) (1995) 2181–2191.
[22] T. Shimada, H. Suzuki, M. Motohara, T. Kuwata, K. Ibuki, M. Ui, T. Iida, M. Fukumoto, T. Miura, M. Hayami. Comparative histopathological studies in the early stages of acute pathogenic and nonpathogenic SHIV-infected lymphoid organs. Virology 306 (2003) 334–346.
[23] J.T. Evans, Y. Okamoto, D.C. Douek, R.D. McFarland, J. Gatlin, R.A. Koup, J.V. Garcia. Thymocyte differentiation from lentivirus-marked CD34(+) cells in infant and adult human thymus. J. Immunol. Methods 245 (2000) 31–43.
[24] K. Yamakami, M. Honda, M. Takei, Y. Ami, N. Kitamura, S. Nishinarita, S. Sawada, T. Horie. Early bone marrow hematopoietic defect in simian/human immunodeficiency virus C2/1-infected macaques and relevance to advance of disease. J. Virol. 78 (2004) 10906–10910.

[25] H. Valentin, M.T. Nugeyre, F. Vuillier, L. Boumsell, M. Schmid, F. Barre-Sinoussi, R.A. Pereira, Two subpopulations of human triple-negative thymic cells are susceptible to infection by human immunodeficiency virus type 1 in vitro, J. Virol. 68 (1994) 3041–3050.

[26] Y. Nishimura, T. Igarashi, O.K. Donau, A. Buckler-White, C. Buckler, B.A. Lafont, R.M. Goeken, S. Goldstein, V.M. Hirsch, M.A. Martin, Highly pathogenic SHIVs and SIVs target different CD4+ T cell subsets in rhesus monkeys, explaining their divergent clinical courses, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 12324–12329.

[27] R.S. Veazey, M. DeMaria, L.V. Chalifoux, D.E. Shvetz, D.R. Pauley, H.L. Knight, M. Rosenzweig, R.P. Johnson, R.C. Desrosiers, A.A. Lackner, Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection, Science 280 (1998) 427–431.

[28] A. Miyake, K. Ibuki, Y. Enose, H. Suzuki, R. Horiuchi, M. Motohara, N. Saito, T. Nakasone, M. Honda, T. Watanabe, T. Miura, M. Hayami, Rapid dissemination of a pathogenic simian/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection, J. Gen. Virol. 87 (2006) 1311–1320.

[29] S.T. Arron, R.M. Ribeiro, A. Gettie, R. Bohm, J. Blanchard, J. Yu, A.S. Perelson, D.D. Ho, L. Zhang, Impact of thymectomy on the peripheral T cell pool in rhesus macaques before and after infection with simian immunodeficiency virus, Eur. J. Immunol. 35 (2005) 46–55.

[30] M.D. Hazenberg, S.A. Otto, E.S. de Pauw, H. Roelofs, W.E. Fibbe, D. Hamann, F. Miedema, T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events, Blood 99 (2002) 3449–3453.

Journal of General Virology (2006), 87, 1311–1320 DOI 10.1099/vir.0.81307-0

Rapid dissemination of a pathogenic simian/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection

Ariko Miyake,[1,2] Kentaro Ibuki,[1] Yoshimi Enose,[1] Hajime Suzuki,[1] Reii Horiuchi,[1] Makiko Motohara,[1] Naoki Saito,[1] Tadashi Nakasone,[3] Mitsuo Honda,[3] Toshiki Watanabe,[2] Tomoyuki Miura[1] and Masanori Hayami[1]

Correspondence
Masanori Hayami
mhayami@virus.kyoto-u.ac.jp

[1]Institute for Virus Research, Laboratory of Primate Model, Experimental Research Center for Infectious Disease, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

[2]Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo 108-8639, Japan

[3]National Institute of Infectious Disease, Tokyo 162-8640, Japan

A better understanding of virological events during the early phase of human immunodeficiency virus 1 (HIV-1) infection is important for development of effective antiviral vaccines. In this study, by using quantitative PCR and an infectious plaque assay, virus distribution and replication were examined in various internal organs of rhesus macaques for almost 1 month after intrarectal inoculation of a pathogenic simian immunodeficiency virus/HIV chimeric virus (SHIV-C2/1-KS661c). At 3 days post-inoculation (p.i.), proviral DNA was detected in the rectum, thymus and axillary lymph node. In lymphoid tissues, infectious virus was first detected at 6 days p.i. and a high level of proviral DNA and infectious virus were both detected at 13 days p.i. By 27 days p.i., levels of infectious virus decreased dramatically, although proviral DNA load remained unaltered. In the intestinal tract, levels of infectious virus detected were much lower than in lymphoid tissues, whereas proviral DNA was detected at the same level as in lymphoid tissues throughout the infection. In the thymus and jejunum, CD4CD8 double-positive T cells were depleted earlier than CD4 single-positive cells. These results show that the virus spread quickly to systemic tissues after mucosal transmission. Thereafter, infectious virus was actively produced in the lymphoid tissues, but levels decreased significantly after the peak of viraemia. In contrast, in the intestinal tract, infectious virus was produced at low levels from the beginning of infection. Moreover, virus pathogenesis differed in CD4 single-positive and CD4CD8 double-positive T cells.

Received 1 July 2005
Accepted 9 January 2006

INTRODUCTION

A better understanding of virological events during the early phase of human immunodeficiency virus 1 (HIV-1) infection is essential for the development of effective vaccines for preventing virus transmission. This is especially true for mucosal infections, which are the major mode of HIV-1 transmission. Moreover, high virus load in the early phase of infection has been reported to correlate with earlier onset of AIDS (Fauci, 1996; Mellors *et al.*, 1995; Schacker *et al.*, 1996). Therefore, data obtained from the early phase of infection would help to define the pathogenesis of HIV-1.

Several non-human primate models have been used to investigate the early phase of HIV-1 infection (Joag *et al.*, 1997; Lu *et al.*, 1998). In some studies using macaques inoculated with *Simian immunodeficiency virus* (SIV) or an SIV/HIV-1 chimeric virus (SHIV) by a mucosal route (i.e. oral, rectal or vaginal), the virus spread to the systemic lymphoid tissues within 3–7 days post-inoculation (p.i.) following replication for a period of time in the local region (Couëdel-Courteille *et al.*, 1999, 2003; Hirsch *et al.*, 1998; Spira *et al.*, 1996; Stahl-Hennig *et al.*, 1999). However, recent studies have shown that the virus can spread more rapidly to the systemic tissues. Hu *et al.* (2000) detected SIV-infected cells in draining lymph nodes within 18 h of intravaginal exposure. Milush *et al.* (2004) showed that SIV spread to systemic lymphoid tissues 1–2 days after oral inoculation. Miller *et al.* (2005) showed that the dissemination of SIV infection to systemic lymphoid tissues occurred within 1–3 days of vaginal inoculation, although virus production

at this site was established later. Furthermore, Veazey *et al.* (1998) reported that the intestinal tract was one of the major sites of SIV replication and $CD4^+$ T cell depletion in the early phase of infection. In a study using SHIV, Harouse *et al.* (1999) suggested that SHIV using CCR5 as co-receptor for virus entry caused a dramatic loss of $CD4^+$ intestinal T cells followed by a gradual depletion in peripheral $CD4^+$ T cells, whereas infection with SHIV using CXCR4 caused a profound loss in peripheral T cells that was not paralleled in the intestine.

The goals of the present study were to investigate the distribution of pathogenic virus in systemic tissues early after mucosal infection and to determine whether these tissues produced infectious virus, which is considered to play a major role in the spread of virus in the body. A pathogenic molecular clone, SHIV-C2/1-KS661c (Shinohara *et al.*, 1999), which uses two major chemokine receptors, CCR5 and CXCR4, as co-receptors for virus entry, was used to inoculate rhesus macaque monkeys intrarectally. Proviral DNA and infectious virus were quantified by quantitative PCR and infectious plaque assay, respectively. Virus load in the infected individuals has usually been quantified by the copy number of virus RNA or DNA using PCR or by the immunodetection of core protein, p24 or p27 (Chun *et al.*, 1997; Sei *et al.*, 1994; Zhang *et al.*, 1999). However, these methods do not differentiate between infectious and non-infectious virus. The infectious plaque assay used in this study quantified infectious virus only (Kato *et al.*, 1998; Miyake *et al.*, 2004). Our results show that the virus spread rapidly to the systemic tissues soon after intrarectal infection. Thereafter, infectious virus was actively produced in the lymphoid tissues, but decreased significantly after the peak of viraemia. In the intestinal tract, lower levels of infectious virus were produced than in lymphoid tissues throughout the infection.

METHODS

Virus. 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) (Shinohara *et al.*, 1999). SHIV-C2/1-KS661c is a molecular clone constructed from the consensus sequence of SHIV-C2/1 (GenBank accession no. AF217181). SHIV-C2/1-KS661c can infect macaque monkeys by intravenous and intrarectal routes and cause precipitous viraemia and drastic $CD4^+$ cell depletion. Virus stock was prepared from supernatant of a human lymphoid cell line, CEMx174, and stored in liquid nitrogen (−190 °C) until use. The $TCID_{50}$ of the virus stock was measured in CEMx174; 20 $TCID_{50}$ was equivalent to one 50 % macaque infectious dose (MID_{50}).

Monkeys and virus inoculation. Ten adult (5- to 8-year-old) rhesus macaques (*Macaca mulatta*), which were of Chinese origin, were used in this study. All monkeys used were treated in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates in the Institute for Virus Research, Kyoto University. Eight monkeys were anaesthetized by intramuscular injection of ketamine chloride and inoculated intrarectally with 2×10^3 $TCID_{50}$ SHIV-C2/1-KS661c. All intrarectal inoculations were done with a paediatric feeding catheter 10 cm from the anus. The catheter was inserted carefully to avoid causing trauma. Two monkeys were euthanized at each of 3 (animals MM301 and MM307), 6 (MM300 and MM309), 13 (MM313 and MM334) and 27 (MM308 and MM310) days p.i. Two monkeys (MM244 and MM314) were used as uninfected controls.

Sample collection. Blood was collected periodically from all monkeys. Peripheral blood mononuclear cells (PBMCs) and plasma were separated from heparinized blood by Percoll (Lymphocyte Separation Solution; Nacalai Tesque) density-gradient centrifugation. Plasma was frozen at −80 °C until use. Complete sets of organs were obtained at the time of euthanasia. Parts of the samples were frozen directly at −80 °C until further use (i.e. 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). Samples of jejunum and rectum were washed in Dulbecco's modified Eagle's medium (DMEM) containing 0·45 mM dithiothreitol, cut into 1 cm^2 pieces and agitated in DMEM medium containing 5 % fetal calf serum (FCS) for 1 h at room temperature. After 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 r.p.m. for 7 min; pellets were then suspended in 30 % Percoll (Pharmacia) and centrifuged at 1800 r.p.m. for 20 min. The resulting pellets were resuspended in 44 % Percoll, layered on 70 % Percoll and centrifuged at 1800 r.p.m. for 20 min. 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 min at room temperature and the supernatants were removed. This step was repeated three times. The fragments were suspended in RPMI 1640 medium (Gibco) containing 10 % FCS and, after agitation for 30 min at room temperature, the supernatants were removed. The fragments were resuspended in RPMI 1640 medium containing 10 % FCS and type II collagenase (0·2 mg ml^{-1}; Sigma) and agitated for 90 min at room temperature. The suspensions (containing LPL) were filtered through glass-wool columns and cells were enriched by Percoll density-gradient centrifugation as described above for iEL. The cells obtained from each organ were used immediately in the infectious plaque assay and flow-cytometry analysis.

Quantification of plasma viral RNA. The viral RNA loads in plasma were determined by quantitative RT-PCR (Suryanarayana *et al.*, 1998). Total RNAs were prepared from plasma with a QIAamp Viral RNA kit (QIAGEN). RT-PCR was performed with a Taqman EZ RT-PCR kit (Perkin Elmer) for the SIV *gag* region using the following primers: SIV2-696F (5′-GGAAATTACCCAGTACAACAA-ATAGG-3′) and SIV2-784R (5′-TCTATCAATTTTACCCAGGCAT-TTA-3′). A labelled probe, SIV2-731T (5′-Fam-TGTCCACCTGCC-ATTAAGCCCG-Tamra-3′; Perkin Elmer), was used for detection of the PCR products. These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems) and analysed by 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. Proviral DNA loads in tissues were determined by quantitative PCR. DNA samples were extracted directly from frozen tissues with a Qiagen DNeasy Tissue kit. PCR was performed with a Taqman PCR Reagent kit (Perkin Elmer) using the same primer set and probe 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 virus was quantified and isolated by using an infectious plaque assay (Kato *et al.*, 1998). An

agarose-gel bilayer containing RPMI 1640 medium was made in plastic culture dishes with a diameter of 100 mm; the lower layer consisted of 12 ml 1·2% agarose (Agarose NA; Pharmacia) and the upper layer consisted of 12 ml 0·4% low gelling-temperature agarose (SeaPlaque Agarose; FMC). 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 M8166 cells (Clapham *et al.*, 1987) were suspended in 3 ml 0·4% low gelling-temperature agarose solution containing the culture medium and the mixture was immediately overlaid on the agarose-gel layer prepared previously. After the gel had hardened, plates were covered with 12 ml 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 on day 10, plates were stained with 2 ml 0·7% MTT for 2 h to count the number of plaques.

Flow-cytometry analysis. The frequencies of $CD4^+$ single-positive and CD4CD8 double-positive T cells in PBMCs and various tissues were examined by flow cytometry. Lymphocytes were treated with anti-CD3 (FN-18–fluorescein isothiocyanate; Biosource), anti-CD4 (Nu-TH/I–phycoerythrin; NICHIREI) and anti-CD8 (SK1–PerCP; Becton-Dickinson) monoclonal antibodies and examined on a FACScan analyser (Becton Dickinson). The absolute number of lymphocytes in the blood was determined by using an automated blood-cell counter (F-820; Sysmex).

RESULTS

Intrarectal infection of macaque monkeys with SHIV-C2/1-KS661c

Eight rhesus macaque monkeys were inoculated intrarectally with SHIV-C2/1-KS661c and two monkeys were euthanized at each of 3, 6, 13 and 27 days p.i. In the two monkeys that were euthanized at 3 days p.i. (MM301 and MM307), plasma viral RNA was not detected in the time between inoculation and euthanasia. However, plasma viral RNA was first detected at 3 days p.i. in one monkey (MM300) and at 6 or 7 days p.i. in five monkeys (MM309, MM313, MM334, MM308 and MM310) (Fig. 1). The plasma viral RNA load of these monkeys reached peak levels, about 10^8 to 5×10^9 copies ml^{-1}, at 13 days p.i. and then decreased, reaching 10^6 copies ml^{-1} at 27 days p.i. $CD4^+$ T-cell counts in peripheral blood of these monkeys started to decrease from day 6 p.i. and were lower than 500 cells μl^{-1} by 13 days p.i. (Fig. 2). These low counts of $CD4^+$ T cells remained at the same levels until 27 days p.i.

Detection of proviral DNA in various tissues early after intrarectal inoculation

To investigate virus distribution to the systemic tissues early after infection, proviral DNAs in various tissues were determined by quantitative PCR. Proviral DNA was already detected at 3 days p.i. in the rectum and distal lymphoid tissues (thymus and axillary lymph node) of one monkey examined at this time (MM301) (Fig. 3). It was also detected in non-lymphoid tissues (kidney and lung; data not shown) at low levels [<20 copies $(\mu g\ DNA)^{-1}$]. These results show that the virus spread quickly to the systemic tissues after intrarectal inoculation. In both monkeys that were examined at 6 days p.i. (MM300 and MM309), proviral

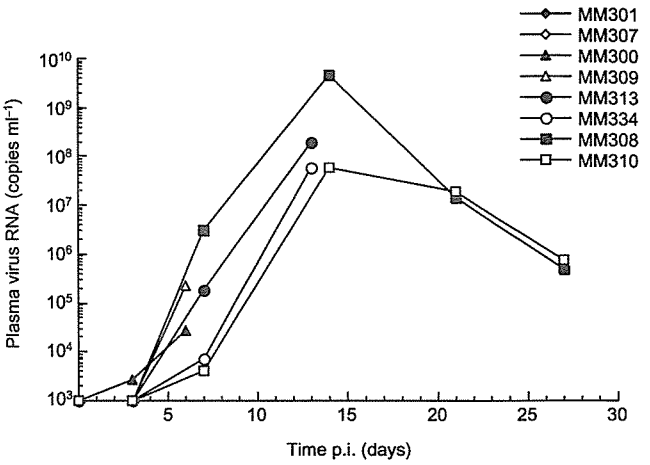

Fig. 1. Plasma viral RNA loads of eight monkeys inoculated intrarectally with SHIV-C2/1-KS661c. MM301 and MM307, MM300 and MM309, MM313 and MM334, and MM308 and MM310 were euthanized at 3, 6, 13 and 27 days p.i., respectively. The detection limit of this assay was 1×10^3 copies ml^{-1}.

DNA was detected in PBMCs as well as other lymphoid tissues (Fig. 3), suggesting that detectable levels of infected cells had drained to the peripheral bloodstream by this time. However, the titres of proviral DNA in these tissues were less than about 10^2 copies $(\mu g\ DNA)^{-1}$ and proviral DNA was not detected in some tissues at 6 days p.i. In monkeys examined at 13 days p.i. (MM313 and MM334), when viraemia reached peak levels, proviral DNA was detected in all tissues examined and virus titres in each tissue were much higher than those determined at 6 days p.i. In most of the lymphoid tissues and the intestinal tract, proviral DNA was detected at $>10^3$ copies μg^{-1} (Fig. 3). In the non-lymphoid tissues of these monkeys, including lung, liver, kidney and

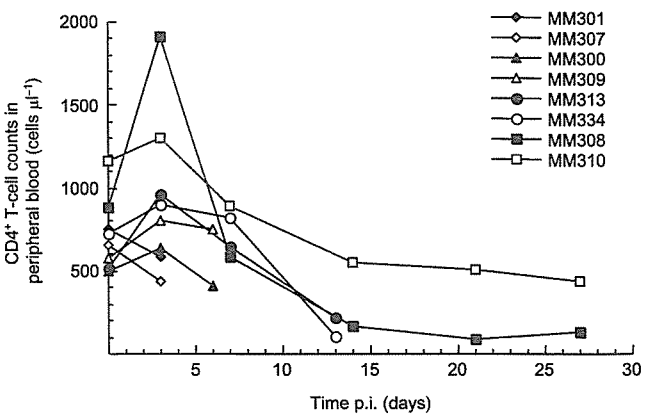

Fig. 2. Number of $CD4^+$ T cells in peripheral blood of eight monkeys inoculated intrarectally with SHIV-C2/1-KS661c. MM301 and MM307, MM300 and MM309, MM313 and MM334, and MM308 and MM310 were euthanized at 3, 6, 13 and 27 days after inoculation, respectively.

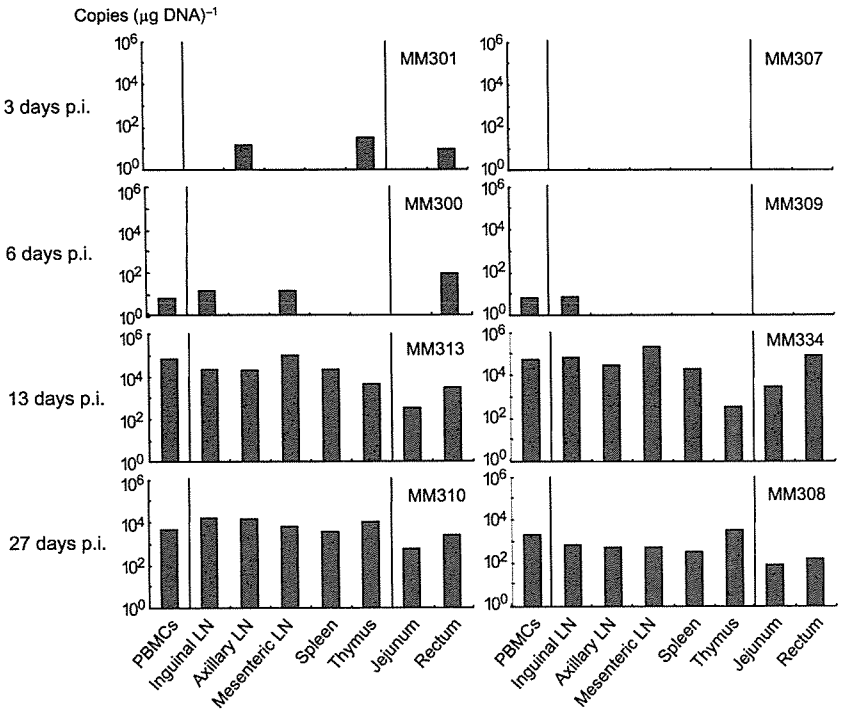

Fig. 3. Proviral DNA loads in various tissues of eight SHIV-C2/1-KS661c-inoculated monkeys. Virus loads were determined by quantitative PCR and are expressed as viral DNA copy numbers (μg total DNA extracted from tissue homogenates)$^{-1}$.

brain, proviral DNA was detected at about 10–10^3 copies μg^{-1} (data not shown). The titres of proviral DNA in all tissues of the monkeys examined at 27 days p.i. (MM308 and MM310) were still $>10^3$ copies μg^{-1} (MM310) or 10^2 copies μg^{-1} (MM308) (Fig. 3). These results show that virus infections in various tissues were amplified from 6 to 13 days p.i. and then decreased, but virus remained detectable in each tissue until 27 days p.i.

Detection of infectious virus in various tissues early after intrarectal infection

Although proviral DNA was present in each tissue early after infection, it was not clear whether these tissues released infectious virus. To observe the release of infectious virus from various tissues, infectious virus only was quantified in these tissues by using an infectious plaque assay. Infectious virus was first detected at 6 days p.i. in inguinal and mesenteric lymph nodes of two monkeys (MM300 and MM309) at 1·0 and 0·5 p.f.u. per 10^6 cells, respectively (Fig. 4). Thereafter, the levels of infectious virus increased dramatically and high titres of infectious virus were detected in many lymphoid tissues of monkeys examined at 13 days p.i. (MM313 and MM334) (Fig. 4). In both of these monkeys, the highest numbers of infectious virus (119 and 100 p.f.u. per 10^6 cells, respectively) were detected in the mesenteric lymph nodes, suggesting that this is the main site of production of infectious virus. Levels of infectious virus in the axillary and inguinal lymph nodes of these monkeys were 55·5–100·0 p.f.u. per 10^6 cells. In MM313, levels of infectious virus in PBMCs and thymus (99·5 and 110·0 p.f.u. per 10^6 cells, respectively) were almost as high as those in the mesenteric lymph node. However, MM334 tissues had remarkably low numbers of infectious virus in PBMCs and thymus (24·0 and 3·5 p.f.u. per 10^6 cells, respectively). In the monkeys examined at 27 days p.i. (MM308 and MM310), infectious virus was detected at very low levels in lymphoid tissues (<19 p.f.u. per 10^6 cells) (Fig. 4), whereas proviral DNA was detected at the same levels as at 13 days p.i. (Fig. 3). These results suggest that, after the peak of viraemia, high levels of virus existed in the lymphoid tissues, but most virus did not replicate there. In particular, PBMCs and thymus contained infectious virus at only 0–5 p.f.u. per 10^6 cells at 27 days p.i., suggesting that these tissues hardly contribute to the release of infectious virus after the peak of viraemia.

In the intestinal tract, infectious virus was hardly detected throughout the infection (Fig. 4). At 13 days p.i., some infectious virus was detected in the jejunum, but titres were much lower than those in the lymphoid tissues (4·5 and 12·5 p.f.u. per 10^6 cells in the jejuna of MM313 and MM334, respectively). These results show that virus replication was much lower in the intestinal tract than in the lymphoid tissues at the early phase of infection, although the virus reached the intestinal tract at the same time that it reached the lymphoid tissues.

Sequential changes in the proportion of $CD4^+$ T cells in various tissues early after intrarectal infection

$CD4^+$ T cells have been reported as the main target and source for amplification of the virus. To estimate the effect of virus replication on the proportion of $CD4^+$ T cells existing in various tissues, sequential changes in the

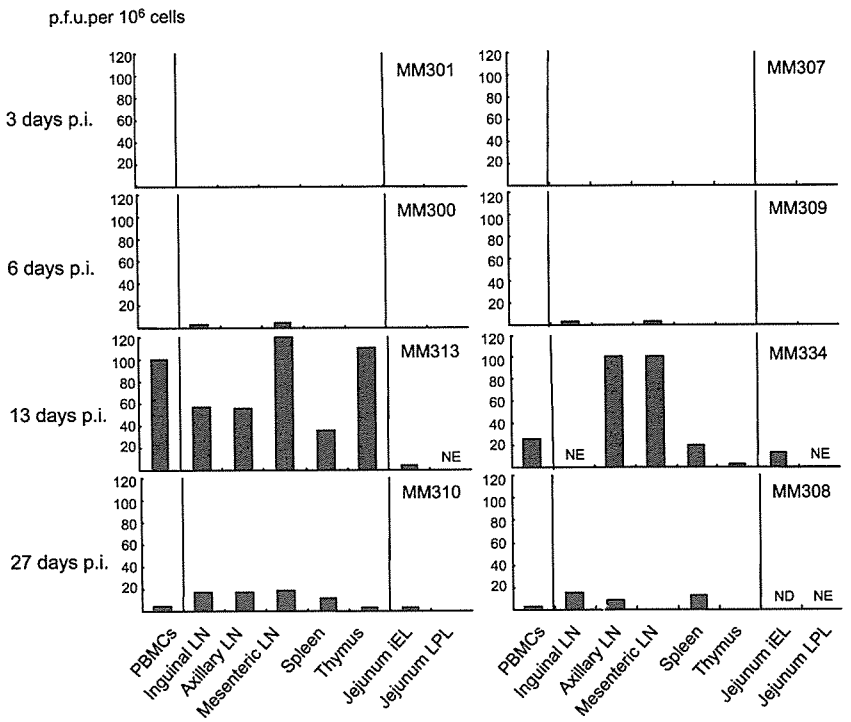

Fig. 4. Infectious virus loads in various tissues of eight SHIV-C2/1-KS661c-inoculated monkeys. Virus loads were determined by infectious plaque assay and are expressed as p.f.u. per 10^6 cells. ND, Assay was not done because not enough lymphocytes were obtained; NE, culture was not evaluated because of contamination.

proportion of $CD4^+$ T cells were examined in each tissue in which virus was detected at various loads by using flow cytometry. The mean percentages of $CD4^+$ T cells in PBMCs, spleen, thymus, and inguinal, axillary and mesenteric lymph nodes of uninfected controls (MM244 and MM314) were 35, 26, 43, 59, 56 and 58 % of total lymphocytes, respectively (Fig. 5). The percentages of $CD4^+$ T cells in PBMCs were higher in monkeys at 6 and 13 days p.i. (62 and 61 % of total lymphocytes, respectively) than in the uninfected normal controls, but then decreased to 12 % of total lymphocytes by 27 days p.i. In other lymphoid tissues, the percentages of $CD4^+$ T cells remained at the level of uninfected normal controls until 6 days p.i. Between 6 and 27 days p.i., the percentages of $CD4^+$ T cells decreased significantly to 9–14 % of whole lymphocytes in each tissue.

In uninfected controls (MM244 and MM314), the percentages of $CD4^+$ T cells in the intestinal tract were lower than those in the lymphoid tissues. iEL and LPL were examined separately, because it was previously reported that the proportions of the major intestinal T-cell subsets differed markedly between the iEL and LPL (Veazey *et al.*, 1997, 2000a, b) and it was expected in the present study that the infection kinetics in iEL and LPL would differ. The mean percentages of $CD4^+$ T cells in the jejuna of control monkeys were 10 % in iEL and 34 % in LPL, and those in the rectums were 5 % in iEL and 11 % in LPL (Fig. 5). $CD4^+$ T cells in the intestinal tract remained at the same level as those in the uninfected controls until 13 days p.i. and then decreased to 1–2 % of whole lymphocytes by 27 days p.i. These sequential changes of $CD4^+$ T cells were almost the same in iEL and LPL of the jejunum and rectum. Thus, SHIV-C2/1 caused marked $CD4^+$ T-cell depletion both in peripheral blood and the intestinal tract. The extent of $CD4^+$ T-cell depletion in intestinal tract and lymphoid tissues correlated with the extent of virus replication in each tissue.

Sequential changes in the proportion of CD4 single-positive (SP) and CD4CD8 double-positive (DP) T cells in the jejunum and thymus

There were larger percentages of CD4CD8 DP T cells in the jejunum than in the lymphoid tissues, apart from the thymus. In the jejuna of the normal control monkeys, the mean percentages of CD4CD8 DP T cells in total $CD4^+$ T cells were 64 % in iEL and 45 % in LPL, whereas in the lymphoid tissues, they were only 8–16 % (data not shown). The proportion of CD4 SP T cells in the jejunum remained at the level of uninfected controls until 13 days p.i. (15 and 34 % in jejunum iEL and LPL, respectively, at 13 days) and then dropped sharply to $<0{\cdot}3$ % in both iEL and LPL by 27 days p.i. This sequential change in the proportion of CD4 SP T cells in the jejunum was the same as that observed for total $CD4^+$ T cells. However, the proportion of CD4CD8 DP T cells started to decrease from day 3 p.i.; at 13 days p.i., it was <5 % in both iEL and LPL of the jejunum (Fig. 6).

In the thymus of the uninfected control monkeys, 91 % of $CD4^+$ T cells were CD4CD8 DP T cells (40 % of total lymphocytes). Moreover, the thymus had many CD3-negative ($CD3^-$) CD4CD8 DP cells (45 % of total lymphocytes). In the thymus, $CD3^+$ CD4CD8 DP cells tended to become depleted first, followed by $CD3^+$ CD4 SP cells and then $CD3^-$ CD4CD8 DP cells (Fig. 7). These results

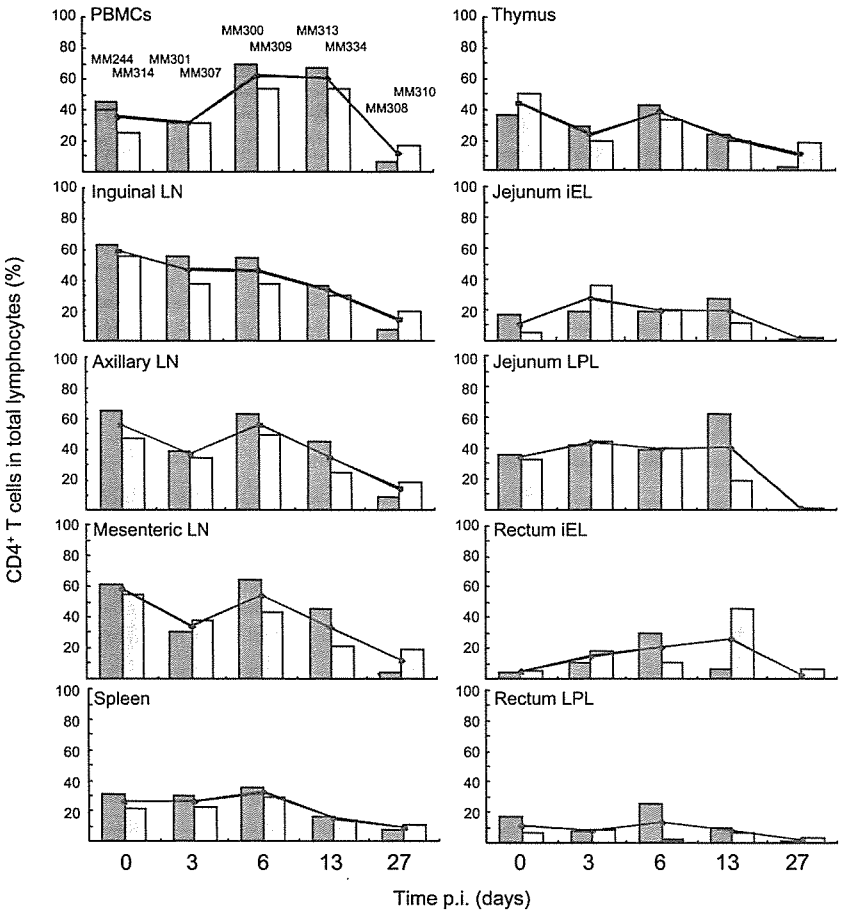

Fig. 5. Sequential changes in the proportion of $CD4^+$ T cells in the various tissues early after intrarectal infection. The percentage of $CD4^+$ T cells in total lymphocytes was determined by flow cytometry. Each bar represents one monkey. The lines indicate the mean values of two monkeys at each time point.

suggest that there is a difference in the effect of virus infection on CD4 SP T cells, CD4CD8 DP T cells and $CD3^-$CD4CD8 DP cells early after intrarectal infection.

DISCUSSION

In this study, to observe the early virological events in various tissues after mucosal infection, SHIV-C2/1-KS661c was used to inoculate rhesus monkeys intrarectally and proviral DNA and infectious viruses were quantified in various tissues by quantitative PCR and infectious plaque assay, respectively. At 3 days p.i., proviral DNA was already present in not only the rectum, but also the thymus, axillary lymph node, kidney and lung. These results suggested that the intrarectally inoculated virus spread quickly to the systemic tissues. Hu *et al.* (2000) showed that SIV penetrated the vaginal mucosa of rhesus macaques within 60 min of intravaginal inoculation, infecting primarily intraepithelial

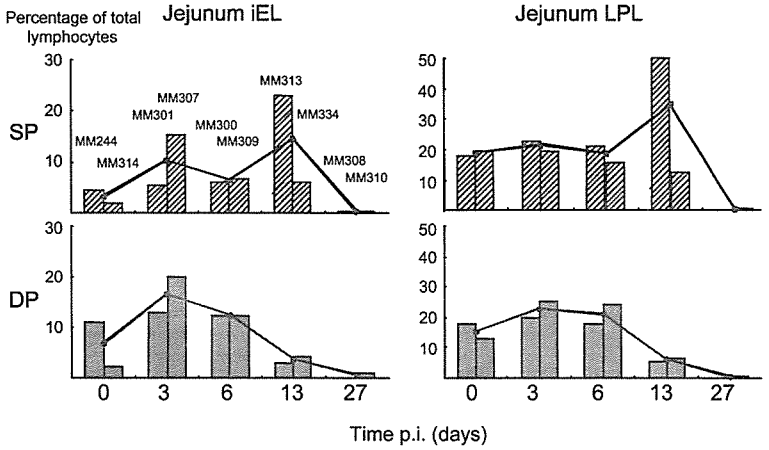

Fig. 6. Sequential changes in the proportion of CD4 single-positive (SP) and CD4CD8 double-positive (DP) T cells in the jejunum iEL and LPL. The percentage of $CD4^+$ T cells in total lymphocytes was determined by flow cytometry. Each bar represents one monkey. The lines indicate the mean values of two monkeys at each time point.

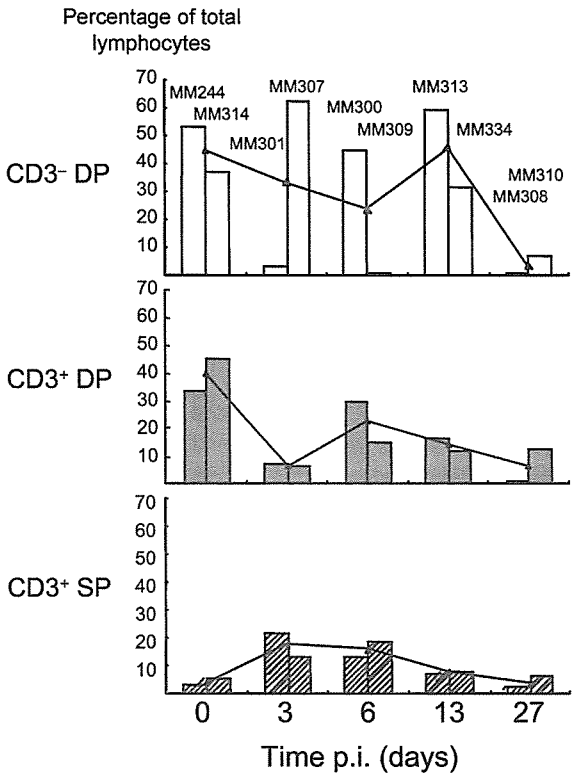

Fig. 7. Sequential changes in the proportion of CD4 single-positive (SP) and CD4CD8 double-positive (DP) T cells in the thymus. The percentage of $CD4^+$ T cells in total lymphocytes was determined by flow cytometry. Each bar represents one monkey. The lines indicate the mean values of two monkeys at each time point.

dendritic cells (DCs), and that SIV-infected cells were detected in iliac lymph nodes within 18 h of inoculation. Because the epithelium of the rectum mucosa is just as rich in DCs as the vaginal mucosa, it appears that the virus is transported to the draining lymph nodes by DCs within several hours of intrarectal exposure in this study. Once the virus reaches the local lymphoid tissues, systemic dissemination might occur shortly thereafter.

Virus levels increased remarkably between 6 and 13 days p.i. and high levels of proviral DNA and infectious virus were detected in various lymphoid tissues at 13 days p.i., which was the time of peak viraemia. Among all the tissues examined, the mesenteric lymph node had the largest level of infectious virus. This result is consistent with a previous study that showed that mesenteric lymph nodes contain numerous SIV-infected cells in the early stages of SIV infection (Cantó-Nogués *et al.*, 2001; O'Neil *et al.*, 1999). The intestinal tract is constantly exposed to antigens in foods and pathogens. Therefore, the mesenteric lymph node, which is a draining lymph node of the intestinal tract, might have many more activated T cells than other lymphoid tissues. Because SIV/HIV-1 can replicate optimally in activated T cells, the mesenteric lymph nodes might release the largest numbers of infectious virus.

After the peak of viraemia, the titre of infectious virus in the lymphoid tissues decreased significantly. Around this time, it is generally recognized that adaptive immunity is induced in the host. Therefore, the induction of such acquired immunity might also result in the suppression of virus replication in the lymphoid tissues. Moreover, $CD4^+$ T cells, which are the main target and source of amplification of the virus (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Sattentau *et al.*, 1988), were depleted in the lymphoid tissues by this time, thus resulting in the low level of virus production there. In contrast, significant proviral DNA remained in the lymphoid tissues after the peak of viraemia. The identity of the cells holding this proviral DNA was not clear, but they might represent a reservoir pool of virus until the development of AIDS.

In the intestinal tract, infectious virus was detected, but the virus load was much lower than in the lymphoid tissues. This is surprising because it was expected that the intestinal tract would have as many activated lymphocytes as the mesenteric lymph node and the virus replicates efficiently in those cells. Some reasons for the low titre of infectious virus in the intestinal tract were considered. Firstly, the sample of intestinal tract, which was separated as iEL and LPL, contains various types of cells and the percentage of $CD4^+$ T cells there was much lower than in samples of lymphoid tissues, thus giving rise to a lower level of virus production in the intestinal tract. In addition, there is a possibility that the intestinal tract has a strong immunity. Following virus infection, the components of innate immunity might respond rapidly and provide time for the subsequent development of adaptive immunity. Natural killer (NK) cells, which are a critical component of innate immunity to virus infection, were reported to mediate suppression of HIV-1 replication by producing CC chemokines or causing cytotoxicity against HIV-1-infected cells (Baum *et al.*, 1996; Fehniger *et al.*, 1998; Kottilil *et al.*, 2003; Levy, 2001; Oliva *et al.*, 1998). In the monkeys used in this study, NK activity using K562 target cells was measured in PBMCs, intestinal tract and inguinal and mesenteric lymph nodes (K. Ibuki, N. Saito, Y. Enose, A. Miyake, H. Suzuki, R. Horiuchi, T. Miura & M. Hayami, unpublished data). Among these tissues, NK activity was much higher in the intestinal tract of both normal and infected monkeys. This result raises the possibility that NK activity in the intestinal tract contributed to the suppression of virus replication in the present study.

In the lymphoid tissues, levels of $CD4^+$ T cells decreased significantly from day 6 p.i. In contrast, in the intestinal tract, $CD4^+$ T cells remained at the same level as in uninfected normal controls until 13 days p.i. These results clearly correlated with the extent of virus replication in the lymphoid tissues and intestinal tract. However, $CD4^+$ T cells in the intestinal tract were finally depleted by 27 days p.i. In previous studies, it was reported that the target tissues or organs of the virus differed when using CXCR4 or CCR5 as

co-receptors of virus entry (Harouse *et al.*, 1999; Reyes *et al.*, 2004). In the early phase of infection, CXCR4-utilizing SHIV causes rapid depletion of $CD4^+$ T cells in the peripheral blood, but not in the intestinal tissues, whereas CCR5-utilizing SHIV causes rapid depletion of $CD4^+$ T cells in the intestinal tissues, but not in the peripheral blood. Recent studies using SIV-infected monkeys showed a profound and selective loss of memory $CD4^+$ $CCR5^+$ T cells in the intestinal tract in the early phase of infection (Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Mattapallil *et al.*, 2005; Li *et al.*, 2005). Moreover, some HIV-1-carrier studies demonstrated that a significant and preferential depletion of mucosal $CD4^+$ T cells that express CCR5 occurs compared with peripheral blood or lymphoid tissues (Veazey *et al.*, 2000a, b; Centlivre *et al.*, 2005). SIV and most primary isolated HIV-1 utilize CCR5 as co-receptor for entry, and target tissues of dual-tropic virus using both CXCR4 and CCR5 were unknown. In this study, it was shown that SHIV-C2/1 using both CXCR4 and CCR5 as co-receptors caused rapid $CD4^+$ T-cell depletion in both peripheral blood and the intestinal tract.

Among the tissues examined, the thymus and intestinal tract had a large percentage of CD4CD8 DP T cells. Both tissues have been reported as sites of maturation of lymphocytes (Haynes *et al.*, 1990; Lundqvist *et al.*, 1995) and CD4CD8 DP T cells have been proposed to be immature T cells. A previous study reported that CD4CD8 DP T cells in the thymus were susceptible to HIV-1 infection (Schnittman *et al.*, 1990). Moreover, CD4 SP and CD4CD8 DP T cells were found to decrease at the same time during acute SIV infection in rhesus macaques in the thymus (Rosenzweig *et al.*, 2000) and intestinal tract (Mattapallil *et al.*, 2000; Smit-McBride *et al.*, 1998). In this study, however, CD4CD8 DP T cells started to decrease earlier than mature CD4 SP T cells in both the thymus and intestinal tract, suggesting that the resultant effect of virus infection is different between the mature and immature T cells in each tissue. CD4CD8 DP T cells were observed to decrease in both tissues before virus replication. This shows that the virus may indirectly kill CD4CD8 DP T cells. Moreover, $CD3^-$ CD4CD8 DP cells, which are precursors of $CD3^+$ CD4CD8 DP cells (Hori *et al.*, 1991), were also depleted in the thymus after the peak of viraemia. Further studies of the pathogenesis of virus infection in immature T cells of the thymus and intestinal tract may lead to better understanding of the mechanisms of $CD4^+$ cell depletion in HIV-1-infected humans.

ACKNOWLEDGEMENTS

We thank James Raymond for the editing of this manuscript. 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.

REFERENCES

Baum, L. L., Cassutt, K. J., Knigge, K. & 7 other authors (1996). HIV-1 gp120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression. *J Immunol* **157**, 2168–2173.

Brenchley, J. M., Schacker, T. W., Ruff, L. E. & 8 other authors (2004). $CD4^+$ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* **200**, 749–759.

Cantó-Nogués, C., Jones, S., Sangster, R. & 8 other authors (2001). *In situ* hybridization and immunolabelling study of the early replication of simian immunodeficiency virus (SIVmacJ5) *in vivo*. *J Gen Virol* **82**, 2225–2234.

Centlivre, M., Sommer, P., Michel, M. & 8 other authors (2005). HIV-1 clade promoters strongly influence spatial and temporal dynamics of viral replication in vivo. *J Clin Invest* **115**, 348–358.

Chun, T.-W., Carruth, L., Finzi, D. & 12 other authors (1997). Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* **387**, 183–188.

Clapham, P. R., Weiss, R. A., Dalgleish, A. G., Exley, M., Whitby, D. & Hogg, N. (1987). Human immunodeficiency virus infection of monocytic and T-lymphocytic cells: receptor modulation and differentiation induced by phorbol ester. *Virology* **158**, 44–51.

Couëdel-Courteille, A., Butor, C., Juillard, V., Guillet, J.-G. & Venet, A. (1999). Dissemination of SIV after rectal infection preferentially involves paracolic germinal centers. *Virology* **260**, 277–294.

Couëdel-Courteille, A., Prétet, J.-L., Barget, N., Jacques, S., Petitprez, K., Tulliez, M., Guillet, J.-G., Venet, A. & Butor, C. (2003). Delayed viral replication and $CD4^+$ T cell depletion in the rectosigmoid mucosa of macaques during primary rectal SIV infection. *Virology* **316**, 290–301.

Dalgleish, A. G., Beverley, P. C., Clapham, P. R., Crawford, D. H., Greaves, M. F. & Weiss, R. A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**, 763–767.

Fauci, A. S. (1996). Host factors in the pathogenesis of HIV disease. *Antibiot Chemother* **48**, 4–12.

Fehniger, T. A., Herbein, G., Yu, H., Para, M. I., Bernstein, Z. P., O'Brien, W. A. & Caligiuri, M. A. (1998). Natural killer cells from $HIV\text{-}1^+$ patients produce C-C chemokines and inhibit HIV-1 infection. *J Immunol* **161**, 6433–6438.

Harouse, J. M., Gettie, A., Tan, R. C. H., Blanchard, J. & Cheng-Mayer, C. (1999). Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. *Science* **284**, 816–819.

Haynes, B. F., Denning, S. M., Le, P. T. & Singer, K. H. (1990). Human intrathymic T cell differentiation. *Semin Immunol* **2**, 67–77.

Hirsch, V. M., Sharkey, M. E., Brown, C. R. & 8 other authors (1998). Vpx is required for dissemination and pathogenesis of SIV_{SM} PBj: evidence of macrophage-dependent viral amplification. *Nat Med* **4**, 1401–1408.

Hori, T., Cupp, J., Wrighton, N., Lee, F. & Spits, H. (1991). Identification of a novel human thymocyte subset with a phenotype of CD3− CD4+ CD8 alpha+beta-1. Possible progeny of the CD3− CD4− CD8− subset. *J Immunol* **146**, 4078–4084.

Hu, J., Gardner, M. B. & Miller, C. J. (2000). Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. *J Virol* **74**, 6087–6095.

Joag, S. V., Adany, I., Li, Z., Foresman, L., Pinson, D. M., Wang, C., Stephens, E. B., Raghavan, R. & Narayan, O. (1997). Animal model of mucosally transmitted human immunodeficiency virus type 1

disease: intravaginal and oral deposition of simian/human immunodeficiency virus in macaques results in systemic infection, elimination of $CD4^+$ T cells, and AIDS. *J Virol* **71**, 4016–4023.

Kato, S., Hiraishi, Y., Nishimura, N., Sugita, T., Tomihama, M. & Takano, T. (1998). A plaque hybridization assay for quantifying and cloning infectious human immunodeficiency virus type 1 virions. *J Virol Methods* **72**, 1–7.

Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J. C. & Montagnier, L. (1984). T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* **312**, 767–768.

Kottilil, S., Chun, T.-W., Moir, S., Liu, S., McLaughlin, M., Hallahan, C. W., Maldarelli, F., Corey, L. & Fauci, A. S. (2003). Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. *J Infect Dis* **187**, 1038–1045.

Levy, J. A. (2001). The importance of the innate immune system in controlling HIV infection and disease. *Trends Immunol* **22**, 312–316.

Li, Q., Duan, L., Estes, J. D. & 7 other authors (2005). Peak SIV replication in resting memory $CD4^+$ T cells depletes gut lamina propria $CD4^+$ T cells. *Nature* **434**, 1148–1152.

Lu, Y., Pauza, C. D., Lu, X., Montefiori, D. C. & Miller, C. J. (1998). Rhesus macaques that become systemically infected with pathogenic SHIV 89.6-PD after intravenous, rectal, or vaginal inoculation and fail to make an antiviral antibody response rapidly develop AIDS. *J Acquir Immune Defic Syndr Hum Retrovirol* **19**, 6–18.

Lundqvist, C., Baranov, V., Hammarstrom, S., Athlin, L. & Hammarstrom, M. L. (1995). Intra-epithelial lymphocytes. Evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. *Int Immunol* **7**, 1473–1487.

Mattapallil, J. J., Reay, E. & Dandekar, S. (2000). An early expansion of $CD8\alpha\beta$ T cells, but depletion of resident $CD8\alpha\alpha$ T cells, occurs in the intestinal epithelium during primary simian immunodeficiency virus infection. *AIDS* **14**, 637–646.

Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M. & Roederer, M. (2005). Massive infection and loss of memory $CD4^+$ T cells in multiple tissues during acute SIV infection. *Nature* **434**, 1093–1097.

Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., Boden, D., Racz, P. & Markowitz, M. (2004). Primary HIV-1 infection is associated with preferential depletion of $CD4^+$ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* **200**, 761–770.

Mellors, J. W., Kingsley, L. A., Rinaldo, C. R., Jr, Todd, J. A., Hoo, B. S., Kokka, R. P. & Gupta, P. (1995). Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Ann Intern Med* **122**, 573–579.

Miller, C. J., Li, Q., Abel, K. & 12 other authors (2005). Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. *J Virol* **79**, 9217–9227.

Milush, J. M., Kosub, D., Marthas, M., Schmidt, K., Scott, F., Wozniakowski, A., Brown, C., Westmoreland, S. & Sodora, D. L. (2004). Rapid dissemination of SIV following oral inoculation. *AIDS* **18**, 2371–2380.

Miyake, A., Enose, Y., Ohkura, S., Suzuki, H., Kuwata, T., Shimada, T., Kato, S., Narayan, O. & Hayami, M. (2004). 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.

Oliva, A., Kinter, A. L., Vaccarezza, M. & 10 other authors (1998). Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. *J Clin Invest* **102**, 223–231.

O'Neil, S. P., Mossman, S. P., Maul, D. H. & Hoover, E. A. (1999). In vivo cell and tissue tropism of SIVsmmPBj14-bcl.3. *AIDS Res Hum Retroviruses* **15**, 203–215.

Reyes, R. A., Canfield, D. R., Esser, U., Adamson, L. A., Brown, C. R., Cheng-Mayer, C., Gardner, M. B., Harouse, J. M. & Luciw, P. A. (2004). Induction of simian AIDS in infant rhesus macaques infected with CCR5- or CXCR4-utilizing simian-human immunodeficiency viruses is associated with distinct lesions of the thymus. *J Virol* **78**, 2121–2130.

Rosenzweig, M., Connole, M., Forand-Barabasz, A., Tremblay, M.-P., Johnson, R. P. & Lackner, A. A. (2000). Mechanisms associated with thymocyte apoptosis induced by simian immunodeficiency virus. *J Immunol* **165**, 3461–3468.

Sattentau, Q. J., Clapham, P. R., Weiss, R. A., Beverley, P. C., Montagnier, L., Alhalabi, M. F., Gluckmann, J. C. & Klatzmann, D. (1988). 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.

Schacker, T., Collier, A. C., Hughes, J., Shea, T. & Corey, L. (1996). Clinical and epidemiologic features of primary HIV infection. *Ann Intern Med* **125**, 257–264.

Schnittman, S. M., Denning, S. M., Greenhouse, J. J., Justement, J. S., Baseler, M., Kurtzberg, J., Haynes, B. F. & Fauci, A. S. (1990). 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.

Sei, S., Kleiner, D. E., Kopp, J. B., Chandra, R., Klotman, P. E., Yarchoan, R., Pizzo, P. A. & Mitsuya, H. (1994). Quantitative analysis of viral burden in tissues from adults and children with symptomatic human immunodeficiency virus type 1 infection assessed by polymerase chain reaction. *J Infect Dis* **170**, 325–333.

Shinohara, K., Sakai, K., Ando, S. & 10 other authors (1999). A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. *J Gen Virol* **80**, 1231–1240.

Smit-McBride, Z., Mattapallil, J. J., McChesney, M., Ferrick, D. & Dandekar, S. (1998). Gastrointestinal T lymphocytes retain high potential for cytokine responses but have severe $CD4^+$ T-cell depletion at all stages of simian immunodeficiency virus infection compared to peripheral lymphocytes. *J Virol* **72**, 6646–6656.

Spira, A. I., Marx, P. A., Patterson, B. K., Mahoney, J., Koup, R. A., Wolinsky, S. M. & Ho, D. D. (1996). Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. *J Exp Med* **183**, 215–225.

Stahl-Hennig, C., Steinman, R. M., Tenner-Racz, K. & 7 other authors (1999). Rapid infection of oral mucosal-associated lymphoid tissue with simian immunodeficiency virus. *Science* **285**, 1261–1265.

Suryanarayana, K., Wiltrout, T. A., Vasquez, G. M., Hirsch, V. M. & Lifson, J. D. (1998). 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.

Veazey, R. S., Rosenzweig, M., Shvetz, D. E., Pauley, D. R., DeMaria, M., Chalifoux, L. V., Johnson, R. P. & Lackner, A. A. (1997). Characterization of gut-associated lymphoid tissue (GALT) of normal rhesus macaques. *Clin Immunol Immunopathol* **82**, 230–242.

Veazey, R. S., DeMaria, M., Chalifoux, L. V. & 7 other authors (1998). Gastrointestinal tract as a major site of $CD4^+$ T cell depletion and viral replication in SIV infection. *Science* **280**, 427–431.

Veazey, R. S., Mansfield, K. G., Tham, I. C., Carville, A. C., Shvetz, D. E., Forand, A. E. & Lackner, A. A. (2000a). Dynamics of CCR5

expression by CD4$^+$ T cells in lymphoid tissues during simian immunodeficiency virus infection. *J Virol* **74**, 11001–11007.

Veazey, R. S., Tham, I. C., Mansfield, K. G., DeMaria, M., Forand, A. E., Shvetz, D. E., Chalifoux, L. V., Sehgal, P. K. & Lackner, A. A. (2000b). Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4$^+$ T cells are rapidly eliminated in early SIV infection in vivo. *J Virol* **74**, 57–64.

Zhang, Z.-Q., Schuler, T., Zupancic, M. & 21 other authors (1999). Sexual transmission and propagation of SIV and HIV in resting and activated CD4$^+$ T cells. *Science* **286**, 1353–1357.

Importance of gastrointestinal ingestion and macromolecular antigens in the vein for oral tolerance induction

Ayako Wakabayashi,[1] Yoshihiro Kumagai,[1] Eiji Watari,[1] Masumi Shimizu,[1] Masanori Utsuyama,[2] Katsuiku Hirokawa[2] and Hidemi Takahashi[1]

[1]*Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan and* [2]*Department of Pathology and Immunology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan*

doi:10.1111/j.1365-2567.2006.02418.x
Received 3 January 2006; revised 15 May 2006; accepted 15 May 2006.
Correspondence: Dr Y. Kumagai, Department of Microbiology and Immunology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113–8602, Japan.
Email: ykumagai@nms.ac.jp
Senior author: Hidemi Takahashi, email: htkuhkai@nms.ac.jp

Summary

Oral administration of a certain dose of antigen can generally induce immunological tolerance against the same antigen. In this study, we showed the temporal appearance of ovalbumin (OVA) antigens in both portal and peripheral blood of mice after the oral administration of OVA. Furthermore, we detected 45 000 MW OVA in mouse serum 30 min after the oral administration of OVA. Based on this observation, we examined whether the injection of intact OVA into the portal or peripheral vein induces immunological tolerance against OVA. We found that the intravenous injection of intact OVA did not induce immunological tolerance but rather enhanced OVA-specific antibody production in some subclasses, suggesting that OVA antigens via the gastrointestinal tract but not intact OVA may contribute to establish immunological tolerance against OVA. Therefore, we examined the effects of digesting intact OVA in the gastrointestinal tract on the induction of oral tolerance. When mice were orally administered or injected into various gastrointestinal organs, such as the stomach, duodenum, ileum, or colon and boosted with intact OVA, OVA-specific antibody production and delayed-type hypersensitivity (DTH) response were significantly enhanced in mice injected into the ileum or colon, compared with orally administered mice. These results suggest that although macromolecular OVA antigens are detected after oral administration of OVA in tolerant-mouse serum, injection of intact OVA cannot contribute to tolerance induction. Therefore, some modification of macromolecular OVA in the gastrointestinal tract and ingestion may be essential for oral tolerance induction.

Keywords: gastrointestinal ingestion; macromolecular antigen; oral tolerance; ovalbumin

Introduction

Although the gastrointestinal tract is incessantly exposed to dietary antigens and commensal micro-organisms, the antigens are not only eliminated, but immunological unresponsiveness to the antigens is also acquired. When an antigen is orally administered to animals, antigen-specific immune responses are suppressed after systemic immunization of the antigen, and this phenomenon is called oral tolerance.[1,2] The development of food hypersensitivity is related to the failure of oral tolerance induction.[3] Food allergy is categorized as class 1 food allergy, which might result from a breach in oral tolerance to foods, or class 2 food allergy, which might

Abbreviations: ABTS, 2,2′-azino-bis diammonium salt; APC, antigen-presenting cell; BSA, bovine serum albumin; CT, cholera toxin; CTM, complete T-cell medium; DC, dendritic cell; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IL, interleukin; i.p., intraperitoneally; IP, immunoprecipitated; M cell, microfold cell; MHC, major histocompatibility complex; IEC, intestinal epithelial cell; MLN, mesenteric lymph node; OVA, ovalbumin; PBS, phosphate-buffered saline; PP, Peyer's patch; SE, standard error; SRBC, sheep red blood cell; TGF, transforming growth factor; Th, T helper; TMB, tetramethyl benzidine.

result from sensitization to respiratory allergens or other sensitization not via gastrointestinal mucosa.[4,5] Class 1 food allergy typically occurs with food proteins, such as eggs or peanuts that are generally stable in digestion, in infants or children.[5] In typical class 2 food allergy, immunoglobulin E (IgE) antibody against respiratory allergens such as pollens recognizes homologous epitopes in food proteins of some fruits or vegetables.[5] In particular, to elucidate the pathogenesis of class 1 food allergy, it is fundamental to clarify mechanisms in the development and failure of oral tolerance. Moreover, the constructive induction of immunological suppression by oral tolerance is expected to contribute to prevent allergy[6] or autoimmune diseases[7,8] in which antigen-specific immune responses are pathologically enhanced.

When a dietary protein antigen is ingested, it is treated by digestive enzymes in the stomach and small intestine. Generated amino acids and small peptides are absorbed via the small intestinal lumen, and enter the portal vein through capillary vessels in the small intestine.[9] However, an antigen that escapes digestion can also enter the body via the intestinal surface. Microfold cells (M cells) over Peyer's patches (PPs) of the intestines take up soluble macromolecule proteins[10–12] as well as viruses[13–15] and bacteria.[16–18] After uptake via M cells, the antigens are processed and presented by dendritic cells (DCs) in PPs.[19] In addition, DCs under intestinal epithelia send dendrites between epithelial cells and directly acquire antigens over epithelial cells.[20] PPs are shown to be inductive sites for oral tolerance where T cells secreting regulatory cytokines, including interleukin (IL)-10[21] and transforming growth factor-β (TGF-β)[22] are induced; however, it is reported that oral tolerance can be induced in mice lacking PPs and mesenteric lymph nodes (MLNs).[23] On the other hand, the liver is shown to be crucial to tolerance induction because the intraportal injection of allogeneic donor cells,[24,25] eggs of a parasite[26] or insoluble protein[27] induces immunological tolerance against the corresponding antigen.

Ovalbumin (OVA) from chicken eggs is a dietary protein antigen that frequently causes food allergy.[28,29] After the oral administration of intact OVA, OVA antigens are known to be detected in peripheral blood and are suggested to contribute to the induction of immunological tolerance against OVA.[30–32] In this study, we attempted to examine OVA antigens in both portal and peripheral blood after the oral administration of OVA and tried to induce tolerance by intraportal and intravenous injection of intact OVA. Furthermore, to investigate the effects of digestion in the gastrointestinal tract on oral tolerance induction, intact OVA molecules were directly injected into the gastrointestinal tract and then the induction profile of tolerance against OVA was assessed.

Materials and methods

Mice

Female BALB/c, C57BL/6 or BDF_1 mice were used between the ages of 6 and 12 weeks. Mice were purchased from Charles River (Tokyo, Japan) or Sankyo Labo Service Co. (Shizuoka, Japan) and maintained in a specific pathogen-free environment.

Oral administration of OVA

OVA, chicken egg, grade V (Sigma, St. Louis, MO) were dissolved in sterilized phosphate-buffered saline (PBS, pH 7·4). Mice were orally administered with 250 ng, 250 μg, 2·5 mg, 25 mg or 250 mg of OVA once. As a control, mice were orally treated with PBS in the same way. In some experiments, mice were orally administered with 1 or 100 mg of OVA or the same dose of OVA plus 10 μg of cholera toxin (CT; List Biological Laboratory Inc., Campbell, CA) once weekly for 4 weeks.

Intraportal or intravenous injection of OVA

Intraportal injection was performed as described previously.[27] Mice were anaesthetized and underwent an abdominal operation. Filtered 2·5 mg or 250 mg of OVA in 250 μl of 0·03% trypan blue-PBS was injected into the portal vein using a 29G needle-tipped syringe. As a control, filtered 0·03% trypan-blue PBS was injected in the same way. In this case, OVA solution was coloured by adding trypan blue to confirm that it was really injected into the liver through the portal vein. After the injection, bleeding from the portal vein was stopped with thrombin (Mochida Pharmaceutical Co., LTD, Tokyo, Japan) and then the peritoneum and skin were sutured.

For intravenous injection, mice were anaesthetized and injected with filtered 2·5 mg or 250 mg of OVA in 250 μl of PBS into the tail vein. As a control, PBS was injected in the same way.

Injection of OVA into the digestive tract

Mice were anaesthetized and underwent an abdominal operation. They were injected with 25 mg of OVA in 250 μl of PBS into the stomach, duodenum, ileum or colon using a 29G needle-tipped syringe, respectively, and then the peritoneum and skin were sutured.

Intraperitoneal immunization of OVA

Mice were intraperitoneally (i.p) injected with 50 μg of OVA and 4 mg of alum, $Al(OH)_3$, in 0·5 ml of PBS. Two weeks later, the second immunization was performed in the same manner. In some experiments, a third boost

was performed by i.p. injection of 0·5 mg of OVA in 0·5 ml of PBS.

Gut content collection

The stomach and small intestine were removed from mice and washed with PBS. Supernatants were collected from the wash fluid and stored frozen at −80° until assay.

Collection of portal or peripheral plasma and faecal samples

For portal blood collection, mice were anaesthetized and underwent an abdominal operation. Portal blood was collected from the portal vein using a 24G catheter and heparinized capillary tubes, and then the peritoneum and skin were sutured. Peripheral blood was collected from anaesthetized mice using heparinized capillary tubes. The blood was centrifuged for 10 min at 6000 *g*, and plasma was collected and stored frozen at −80° until assay.

Faecal extracts were prepared by the method described previously.[33] Fresh faeces were collected and weighed, and PBS containing 0·01% sodium azide was added to the faeces (100 mg/ml). The faeces in PBS were homogenized by continuous shaking for 10 min with a Vortex, and centrifuged for 10 min at 12 000 *g* at 4°. Supernatants were collected and stored frozen at −80° until assay.

Enzyme-linked immunosorbent assay (ELISA)

OVA antigen levels in the gut contents or the plasma and anti-OVA antibody levels in the plasma or the faecal extracts were determined by ELISA as described previously.[34,35] For the assay of OVA antigen levels, 96-well flat-bottomed microtitre plates were coated with rabbit anti-OVA IgG (Rockland, Gilbertsville, PA) in carbonate buffer (pH 9·6) at 4° overnight. Wells were blocked with 1% bovine serum albumin (BSA) in PBS at 37° for 1 hr. Gut contents or plasma samples diluted appropriately in PBS were added to the wells in duplicate, and incubated at 37° for 1 hr. Biotinylated anti-OVA IgG (Rockland) was added to the wells and incubated at 37° for 1 hr. Horseradish peroxidase-conjugated streptavidin (Caltag Laboratory, Burlingame, CA) was then added and incubated at 37° for 30 min. Enzyme reaction was performed with 1 mM 2,2′-azino-bis diammonium salt (ABTS, Sigma) in sodium citrate buffer (pH 5·4) in the presence of 0·01% H_2O_2. The reaction was interrupted by the addition of 2 mM NaN_3 in PBS, and absorbance was measured at 415 nm. To draw a standard curve, various quantities of OVA were added to the part of the plate coated with anti-OVA IgG and blocked. The wells were added to biotinylated anti-OVA IgG and coloured in the same manner as the sample wells. For the assay of anti-OVA immunoglobulin, plates were coated with OVA (100 µl of 1 mg/ml) in carbonate buffer. After blocking, diluted plasma or faecal extract samples were added to the wells and incubated. Biotinylated anti-mouse immunoglobulins (Amersham Life Science, Amersham, UK), IgA (Sigma), IgG1, IgG2a, IgG2b, IgM or IgE (BD PharMingen, San Diego, CA) were added to the wells and incubated. Horseradish peroxidase-conjugated streptavidin was then added and incubated. Enzyme reaction was performed with ABTS, and absorbance was measured at 415 nm. To draw a standard curve, part of the assay plate was coated with various quantities of purified mouse IgG1, IgG2a (BD PharMingen) or IgA (ICN/Cappel, Aurora, OH). After blocking, biotinylated antimouse IgG1, IgG2a or IgA were added to the wells and coloured in the same manner as the sample wells.

Immunoprecipitation and immunoblotting

For immunoprecipitation, 0·1 mg of rabbit anti-OVA IgG (Rockland) was incubated with 20 µl of protein-G coupled sepharose (Sigma) at 4° on an orbital shaker overnight. After washing with PBS three times, the treated sepharose was incubated with 1 ml of mouse serum or 100 ng of OVA mixed with 1 ml of untreated mouse serum at 4° on an orbital shaker overnight. After washing with PBS twice and 0·05 M Tris buffer once, the sepharose was resuspended in 20 µl of sample buffer (Invitrogen, Carlsbad, CA) including sample reducing agent (Invitrogen) and heated at 70° for 10 min. The supernatants were collected and diluted fivefold with the sample buffer. Five µl of the diluted samples were loaded on 4–12% Bis-Tris gel (Invitrogen). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen). The membranes were blocked in 1% BSA 0·1% Tween-20 PBS and incubated with rabbit anti-OVA polyclonal IgG (Rockland) at 4° overnight. This was followed by incubation with peroxidase-conjugated anti-rabbit IgG (Seikagaku Corporation, Tokyo, Japan). The tetramethyl benzidine (TMB) substrate kit for peroxidase (Vector Labotarories, Inc., Burlingame, CA) was used for detection.

OVA-specific T cell proliferation

The spleens or MLNs were removed and crushed in RPMI-1640 medium (Sigma). Red blood cells in spleen cells were depleted by cell lysis. Single spleen cells or MLN cells were suspended in complete T-cell medium (CTM) composed of RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0·1 mM nonessential amino acid, a mixture of vitamins, 1 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (FCS). OVA-specific T-cell proliferation was analysed by the modified method described previously.[36] Spleen or MLN T cells were taken using a nylon wool column and a single cell suspension was

prepared in the CTM. The spleen or MLN T cells (5×10^5) were cultured with 0, 111, 333 or 1000 mg/ml of OVA in the presence of $2{\cdot}5 \times 10^5$ of irradiated (3000 rad) spleen cells from naive C57BL/6 mice in 96-well flat-bottomed culture plates at 37° in 5% CO_2 for 4 days. During the last 18 hr of the 4-day culture, 0·5 μCi of tritiated [^{3}H]thymidine was added to each well. The plates were harvested and counted using a β counter (1450 Microbeta Trilux; Wallac, Gaithersburg, MD).

Cytokine analysis

Levels of IL-4 or IL-2 in the spleen T-cell culture supernatants were analysed by an IL-4-dependent cell line, CT.4S cells (kindly gifted by Prof. William E. Paul, National Institutes of Health, Bethesda, MD), or an IL-2-dependent cell line, CTLL-2 cells (American Type Culture Collection, ATCC, Manassas, VA). T-cell culture supernatants were collected on day 3 and stored frozen at −80° until assay. CT.4S or CTLL-2 cells (5×10^3) were cultured in the presence of the supernatant in 96-well flat-bottomed culture plates at 37° in 5% CO_2 for 3 or 2 days, respectively. To draw a standard curve, CT.4S or CTLL-2 cells were cultured with various quantities of recombinant mouse IL-4 (rIL-4) or rIL-2 (Genzyme, Cambridge, MA), respectively. During the last 18 hr of incubation, 0·5 μCi of [^{3}H]thymidine was added to each well. The plates were harvested and counted using the β counter.

Delayed-type hypersensitivity (DTH) response

Mice were anaesthetized and intradermally injected 20 μg of OVA in 20 μl of saline into the right ears. As negative controls, saline was injected into the left ears. Ear thickness was measured using a dial thickness gauge (Ozaki MFG. Co., LTD. Tokyo, Japan) before and 24 hr after the injection. The swelling rate of the ear was calculated as follows: [(thickness of the ear 24 hr after challenge − thickness of the ear before challenge)/thickness of the ear before challenge] × 100%.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences between groups. Data were considered significant at $P < 0{\cdot}05$.

Results

Appearance of detectable OVA antigens in both portal and peripheral blood after oral administration of OVA

The oral administration of OVA induces oral tolerance against OVA, and it has been shown that OVA antigens

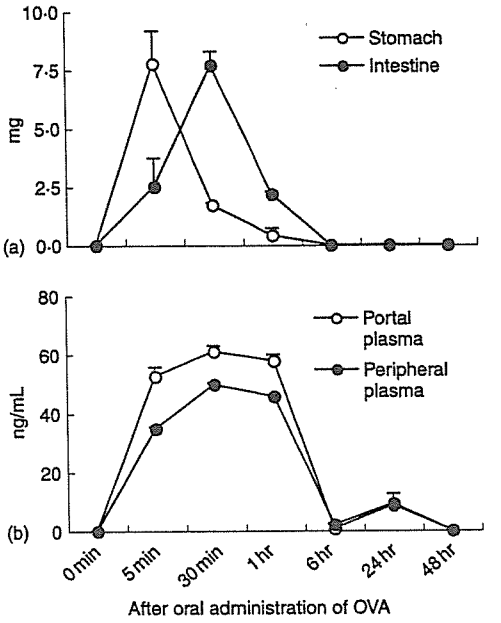

Figure 1. Kinetics of OVA antigens detected in gut contents and plasma after oral administration of OVA. BALB/c mice were orally administered with 25 mg of OVA. At various times after oral administration, the stomach or small intestine contents were collected (a). Portal and peripheral blood was collected from mice at various times after oral administration (b). Levels of OVA antigens in the gut contents or plasma were assessed by ELISA. The data are expressed as the mean + standard error (SE) of three mice.

are detected in peripheral blood after the oral administration of OVA.[30,31] First, we attempted to detect OVA antigens in the digestive tract and in blood after absorption via the guts.

When 25 mg of OVA was orally administered to mice, many OVA antigens in the gastric contents and fewer OVA antigens in the small intestinal contents were detected at 5 min after oral administration (Fig. 1a). While the amount of detectable OVA antigens was reduced at 30 min in the stomach, they reversely increased in the small intestine at the same time. OVA antigens in the guts could not be detected at 6 hr and subsequently (Fig. 1a).

OVA antigens were observed in both portal and peripheral blood at 5 min after the oral administration of OVA (Fig. 1b). They reached a peak at 30 min and became undetected at 6 hr after oral administration (Fig. 1b). Thus, kinetics of OVA antigens in the portal and peripheral blood corresponded to that in the small intestinal contents. The amount of OVA antigens detected in the portal and peripheral blood was dependent on the dose of orally administered OVA (Fig. 2). Levels of OVA antigens in the portal blood were more than in the peripheral blood (Fig. 1b and Fig. 2). OVA antigens were observed in the blood from all strains used in this study, BALB/c, C57BL/6, and BDF_1 mice (data

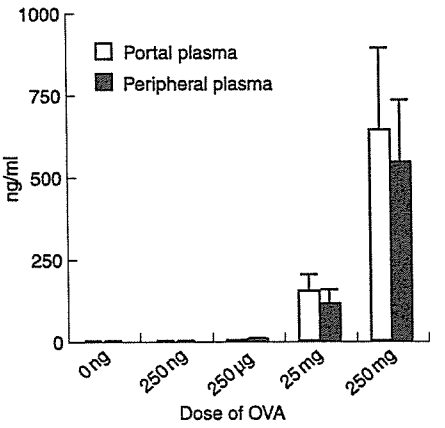

Figure 2. Level of OVA antigens in plasma is dependent on the dose of orally administered OVA. BALB/c mice were orally administered with various doses of OVA. Portal and peripheral blood was collected from mice at 30 min after oral administration. Levels of OVA antigens in plasma were assessed by ELISA. The data are expressed as the mean + SE of three mice.

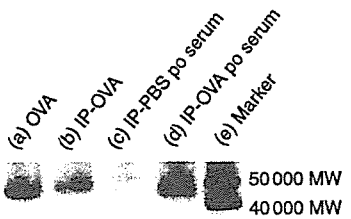

Figure 3. Immunoblot of OVA antigens in the serum after the oral administration of OVA. C57BL/6 mice were orally administered with 250 mg of OVA or PBS. Blood was collected from the heart 30 min after the oral administration (po) of OVA or PBS. OVA antigens in the serum were detected by immunoprecipitation and immunoblotting. Immunoprecipitation was performed using a rabbit anti-OVA polyclonal antibody on serum from mice or OVA mixed with serum from untreated mice. Immunoprecipitated (IP) samples or 2·5 ng of OVA were loaded. Proteins were transferred to a PVDF membrane and detected using the anti-OVA polyclonal antibody. (a) 2·5 ng of OVA, (b) IP sample of OVA mixed with untreated mouse serum, (c) IP sample of the serum after oral administration of PBS, (d) IP sample of the serum after the oral administration of OVA, (e) molecular markers.

not shown). To examine the molecular weights of OVA antigens in the serum, we performed immunoprecipitation and immunoblotting in serum samples using an anti-OVA polyclonal antibody. Surprisingly, 45 000 MW OVA was clearly detected in the serum 30 min after the oral administration of OVA (Fig. 3). In our immunoprecipitation and immunoblotting system using an anti-OVA polyclonal antibody, digested OVA fragments could not be detected in the serum. These results suggest that the macromolecules, 45 000 MW OVA antigens, were absorbed via the small intestines, transferred to the portal vein and circulated in the bloodstream in normal mice, in which oral tolerance was induced.

OVA antigens become undetected in the blood with an increase in mucosal and systemic OVA-specific immunoglobulin

Next, we attempted to analyse OVA antigens in blood from mice induced with OVA-specific immune responses by the oral administration of OVA plus CT adjuvant. Mice were orally administered with 1 or 100 mg of OVA or the same dose of OVA plus CT every week. Peripheral blood samples from mice were collected 30 min after oral administration every week and levels of OVA antigens and OVA-specific IgG in the blood were assessed by ELISA. The production of OVA-specific faecal IgA, OVA-specific T-cell proliferation, and secretion of cytokines were also analysed in mice.

The production of OVA-specific plasma IgG1 and faecal IgA was enhanced after the oral administration of 1 or 100 mg of OVA plus CT; however, the oral administration of OVA without CT did not induce OVA-specific systemic and mucosal antibody production (Fig. 4). OVA-specific serum IgG2a, T helper (Th)1-type antibody, could not be detected by oral administration with and without CT during the experimental period. As shown in Fig. 5, OVA-specific T-cell proliferation was also observed in spleen T cells and MLN T cells from mice orally administered with OVA plus CT (Fig. 5a). IL-4 secretion was observed in culture supernatants from proliferated spleen T cells; however, IL-2 was not

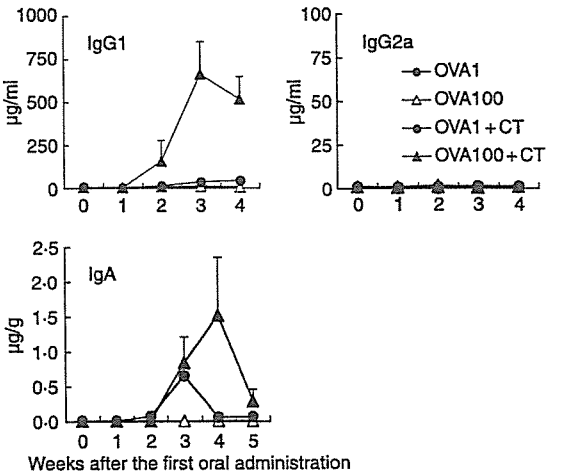

Figure 4. Production of OVA-specific systemic IgG and faecal IgA by oral administration of OVA plus CT. C57BL/6 mice were orally administered with 1 or 100 mg of OVA, or the same dose of OVA plus CT once weekly for 5 weeks. Peripheral blood and faeces were collected from the mice every week. OVA-specific plasma IgG1 and IgG2a and faecal IgA were assessed by ELISA. The data are expressed as the mean anti-OVA immunoglobulin in plasma (μg/ml) or faeces (μg/g) + SE of four mice.

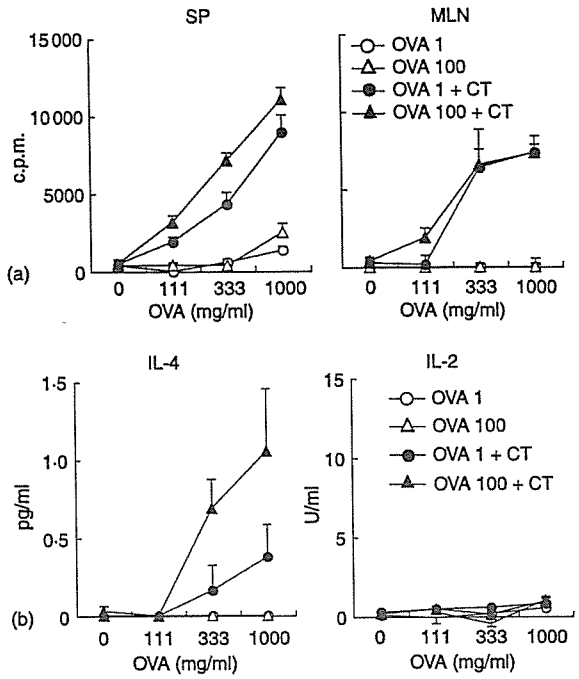

Figure 5. OVA-specific T cell proliferation and secretion of Th2-type cytokine by oral administration of OVA plus CT. C57BL/6 mice were orally administered with 1 mg or 100 mg of OVA, or the same dose of OVA plus CT once weekly for 3 weeks. Spleens and MLNs were removed 2 weeks after the third administration and T cells were isolated. T cells from the spleen or MLN were cultured with 0, 111, 333 or 1000 mg/ml of OVA in the presence of irradiated spleen cells from naive C57BL/6 mice for 4 days. OVA-specific T cell proliferation was assessed as described in Materials and methods (a). Splenic T cell culture supernatants were collected on day 3. IL-4-dependent cell line, CT-4S, or IL-2-dependent cell line, CTLL-2, were cultured in the presence of the supernatant for 3 or 2 days, respectively. Cytokine levels were assessed as described in Materials and methods (b). The results are expressed as the level of orally administered mice minus the level of normal mice. The results are shown as the mean + SE in triplicate. Data are representative of two separate experiments.

detected (Fig. 5b). These results demonstrate that the oral administration of OVA plus CT induces mucosal IgA and Th2-type systemic immune responses.

Although OVA antigens in the blood were sufficiently detected at 1 week after the oral administration of 100 mg of OVA plus CT, they were remarkably reduced at 2 weeks and subsequently remained reduced (Fig. 6), while OVA-specific systemic IgG1 and mucosal IgA were produced (Fig. 4). On the other hand, in mice orally administered with 100 mg of OVA, OVA antigens were detected every week. When 1 mg of OVA or the same dose of OVA plus CT was orally administered, OVA antigens were undetectable in the blood (Fig. 6).

These results show that in immunized mice, mucosal anti-OVA IgA may bind OVA antigens in the gastrointestinal tract and block OVA from entering into the mucosal tissues. Furthermore, OVA antigens may be caught by

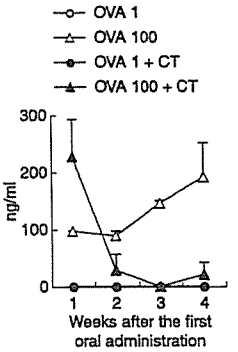

Figure 6. OVA antigens become undetectable after oral administration of OVA plus CT. C57BL/6 mice were orally administered with 1 or 100 mg of OVA or the same dose of OVA plus CT once weekly for 4 weeks. Peripheral blood was collected from the mice 30 min after oral administration every week. OVA antigens in plasma were assessed by ELISA. The data are expressed as the mean + SE of three mice.

anti-OVA IgG and immune complexes might be undetected in ELISA.

Oral administration of intact OVA suppresses OVA-specific immune responses whereas intraportal or intravenous injection cannot induce immunological suppression

The results indicate that OVA antigens appear in the blood from mice in which oral tolerance is induced, although they are markedly reduced in mice in which OVA-specific immune responses are induced. Therefore, we attempted to induce immunological tolerance against OVA by the intravenous injection of OVA. Mice were orally administered or injected into the portal or peripheral vein with 2·5 mg or 25 mg of OVA, and then i.p. immunized with OVA plus alum. OVA-specific systemic immunoglobulin production and DTH response were assessed in the mice.

The oral administration of a high dose of intact OVA significantly suppressed DTH response against OVA ($P < 0{\cdot}005$), compared with that in the oral treatment of PBS (Fig. 7a). The DTH response, however, was not significantly suppressed by the intraportal or intravenous injection of intact OVA (Fig. 7b, c). When control PBS was injected into ears, DTH response was not induced (Fig. 7).

Oral administration of OVA also significantly suppressed the production of anti-OVA immunoglobulins (Fig. 8a), whereas the production was significantly enhanced rather than suppressed at 1 week after the intraportal or intravenous injection of OVA (Fig. 8b, c). All immunoglobulin subclasses, IgG1, IgG2a, IgG2b, IgM and IgE, were significantly suppressed by the oral administration of OVA (Fig. 8a). On the other hand, in mice injected with OVA intraportally or intravenously, anti-OVA IgG1

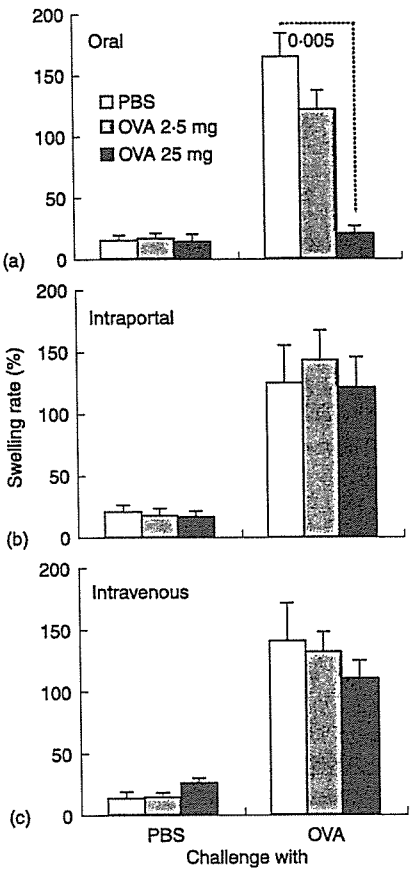

Figure 7. OVA-specific DTH response is suppressed by oral administration of intact OVA, but not by intraportal or intravenous injection of intact OVA. BDF_1 mice were orally administered (a) or injected into the portal (b) or peripheral vein (c) with 2·5 mg or 25 mg of OVA or PBS, and then i.p. immunized with OVA plus alum twice. DTH response was assessed 2 weeks after the second immunization of OVA plus alum. Ear thickness was measured 24 h after challenge of OVA or PBS into the ear. The data are expressed as the mean + SE of five to six mice. The value represents statistical significance ($P <$) compared with control mice treated with PBS in the same way.

at 1 week, IgG2a, IgG2b and IgM were significantly enhanced, although IgG1 at 4 weeks and IgE were suppressed (Fig. 8b, c).

The data clearly show that the injection of intact OVA into the portal or peripheral vein cannot induce immunological tolerance but rather enhances part of immune responses against OVA.

The injection of intact OVA into the lower intestinal tract is less effective in terms of OVA-specific tolerance induction

We therefore investigated whether the modification of intact OVA by gastrointestinal digestion was essential to induce oral tolerance. Mice were orally administered or injected into the guts, stomach, duodenum, ileum, or colon, with 25 mg of intact OVA. OVA-treated and untreated mice were i.p. immunized with OVA plus alum twice, and then OVA-specific plasma immunoglobulin and DTH response were assessed.

OVA-specific immunoglobulin production was remarkably suppressed by the oral administration of intact OVA, whereas immunoglobulin was sufficiently produced in untreated mice (Fig. 9a). The injection of intact OVA into the stomach, duodenum, ileum, or colon significantly enhanced OVA-specific immunoglobulin production ($P < 0·05$), compared with the oral administration of intact OVA (Fig. 9a). DTH response against OVA was also significantly enhanced in mice injected with intact OVA into the ileum or colon ($P < 0·05$ or 0·005, respectively), compared with the oral administration of OVA (Fig. 9b). Levels of OVA-specific immunoglobulins and DTH responses were higher in mice injected with intact OVA into the lower intestinal tract.

The results demonstrate that the appropriate digestion of intact OVA in the gastrointestinal tract is crucial for the induction of oral tolerance against OVA.

Discussion

In this study, we showed that after the oral administration of OVA, OVA antigens were absorbed via the small intestines, transferred into the liver via the portal vein, and circulated in the bloodstream. This suggests that digestive enzymes do not completely digest OVA to amino acids and small peptides that do not have antigenicity, and OVA antigens can cross the intestinal surface.

M cells that exist over PPs uptake soluble macromolecule proteins.[10–12] OVA antigens may be taken up by M cells at the intestinal surface and then they may be processed and presented by immature DCs in PPs, which are shown to be inductive sites for oral tolerance where T cells secreting regulatory cytokines, IL-10[21] and TGF-β,[22] are induced. DCs may capture OVA antigens, present antigen epitopes to naïve T cells, and induce regulatory T cells in PPs. M cells are shown to also exist in small intestinal villi,[37] suggesting that antigens may be taken up by M cells apart from PPs. Moreover, DCs may send dendrites between epithelial cells and acquire OVA antigens over epithelia. Thus, some of the OVA antigens that cross the intestinal surface are captured by DCs in these intestinal mucosal tissues.

A protein antigen is digested by digestive enzymes to amino acids and small peptides.[9] They are absorbed via epithelial cells, and enter the portal vein through capillary vessels in the small intestine. After the oral administration of OVA to mice, OVA antigens are detected in peripheral blood.[30,31] Furthermore, our results clearly showed that OVA antigens, 45 000 MW of proteins, enter the portal vein and then the bloodstream after the oral administration of OVA. OVA antigens taken up by M cells, or using other routes, may enter the portal vein via capillary