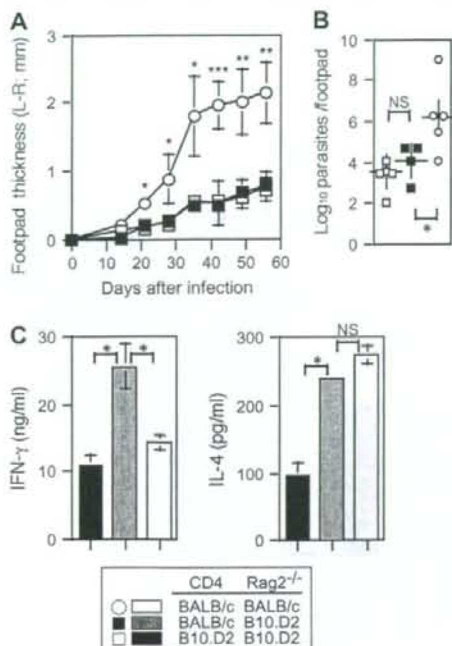

Fig. 2. The outcome of *Leishmania major* infection on Rag-$2^{-/-}$ mice reconstituted with $CD4^+$ T cells is determined by the genetic background of Rag-$2^{-/-}$ recipients. B10.D2-Rag-$2^{-/-}$ mice were reconstituted with 10^7 naive $CD4^+$ T cells collected from either B10.D2 (open squares and closed bars) or BALB/c (closed squares and gray bars). BALB/c-Rag-$2^{-/-}$ mice were reconstituted with BALB/c $CD4^+$ T cells (open circles and open bars). Three days after reconstitution, mice were administered with *L. major* promastigotes. (A) Footpad swelling. Statistical significance was observed in closed squares versus open circles. (B) Parasite burdens in infected footpads. +, mean of each group. (C) IFN-γ and IL-4 production from splenocytes of *L. major*-infected mice upon stimulation with parasite antigens. Although not shown, no $CD8^+$ T cells or B cells were detected in the spleens of Rag-$2^{-/-}$ mice reconstituted with $CD4^+$ T cells. Statistical significance: NS, not significant; $^*P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$.

As shown in Fig. 3(A–C), the outcome of disease was largely dependent on the genetic background of parasitized cells. Upon inoculation with parasitized B10.D2-Rag-$2^{-/-}$ splenocytes, both B10.D2 and BALB/c WT mice exhibited a milder symptom (Fig. 3A and B) and a T_h1-biased response (Fig. 3C) as compared with BALB/c WT mice inoculated with parasitized BALB/c-Rag-$2^{-/-}$ splenocytes. These results indicate that BM-derived non-T and -B cells in Rag-$2^{-/-}$ splenocytes that initially encounter

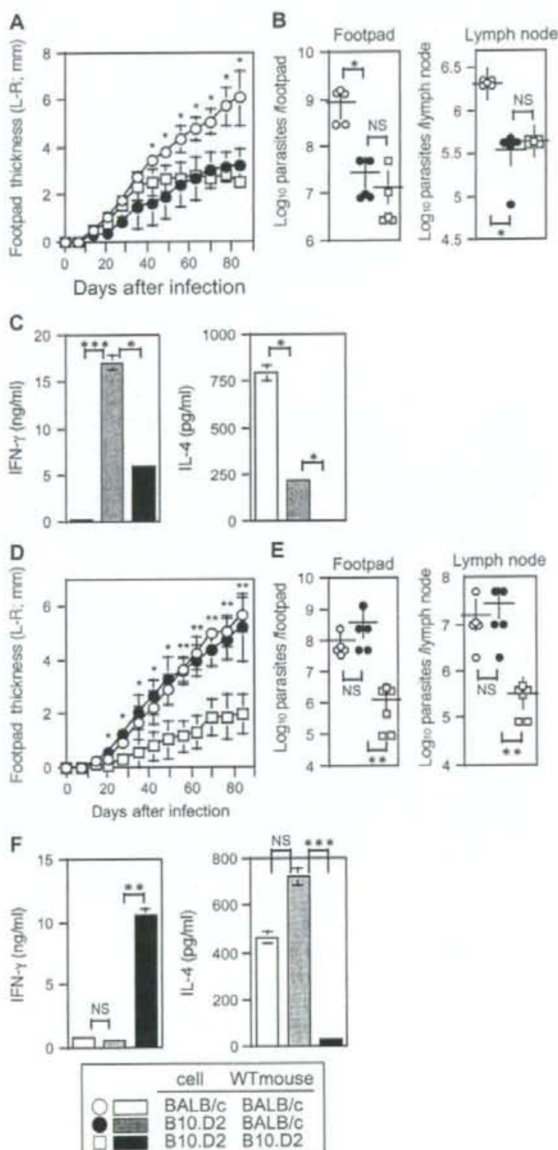

Fig. 3. (A–C) The genetic background of *Leishmania major*-parasitized Rag-$2^{-/-}$ splenocytes determines the outcome of *L. major* infection on WT BALB/c mice. BALB/c WT mice were inoculated with 10^7 parasitized BALB/c-Rag-$2^{-/-}$ (open circles and open bars) or B10.D2-Rag-$2^{-/-}$ (closed circles and gray bars) splenocytes. B10.D2 WT mice were inoculated with 10^7 parasitized B10.D2-Rag-$2^{-/-}$ splenocytes (open squares and closed bars). (D–F) The depletion of $CD11c^+$ DCs from *L. major*-parasitized Rag-$2^{-/-}$ splenocytes impairs the ability to determine the outcome of *L. major* infection. BALB/c WT mice were inoculated with 10^7 DC-depleted parasitized BALB/c-Rag-$2^{-/-}$ splenocytes (open circles and open bars) or DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes (closed circles and gray bars). B10.D2 WT mice were inoculated with 10^7 DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes (open squares and closed bars). (A and D) Footpad swelling caused by *L. major* infection. (B and E) Parasite burdens in footpads and popliteal lymph nodes of infected legs. +, mean of each group. (C and F) IFN-γ and IL-4 production from *L. major*-infected mouse splenocytes upon stimulation with parasite antigens. Statistical significance: NS, not significant; $^*P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$. Asterisks indicated in (A) are open circles versus closed circles and in (D) are closed circles versus open squares.

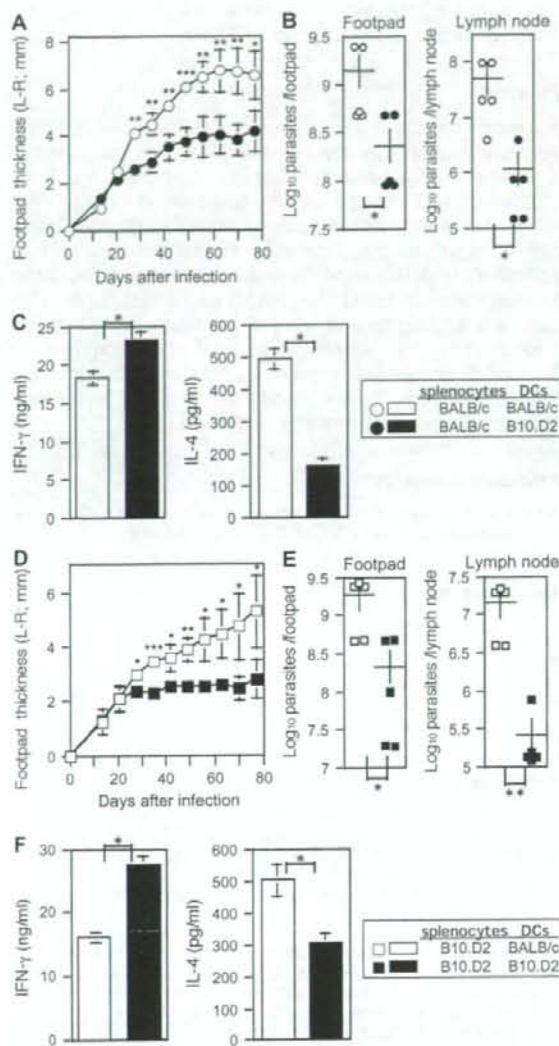

Fig. 4. *Leishmania major*-parasitized Rag-$2^{-/-}$ splenocytes mixed with parasitized B10.D2 CD11c$^+$ DCs lead BALB/c WT mice to mild outcome of the disease and T_h1 response. (A–C) DC-depleted *L. major*-parasitized BALB/c-Rag-$2^{-/-}$ splenocytes were mixed with parasitized CD11c$^+$ DCs from BALB/c (open circles and open bars) or B10.D2 (closed circles and closed bars). (D–F) DC-depleted *L. major*-parasitized B10.D2-Rag-$2^{-/-}$ splenocytes were mixed with parasitized CD11c$^+$ DCs from BALB/c (open squares and open bars) or B10.D2 (closed squares and closed bars). Parasitized cells (10^7 per mouse) were inoculated into BALB/c mice. (A and D) Footpad swelling caused by *L. major* infection. (B and E) Parasite burdens in footpads and popliteal lymph nodes of infected legs. +, mean of each group. (C and F) IFN-γ and IL-4 production from splenocyte stimulation with parasite antigens. Statistical significance: $^*P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$.

parasites determine the outcome of infection including footpad swelling, parasite burden and immune responses upon *L. major* infection. Those cells likely include macrophages and DCs.

Genetic background of CD11c$^+$ DCs determines outcome of disease

L. major promastigotes are able to penetrate into DCs in addition to macrophages (16–18). Since DCs are known to be the only antigen-presenting cells (APCs) capable of activating naive T cells (19), they are likely candidates determining the immune response upon *L. major* infection. To this end, CD11c$^+$ DCs were removed from *L. major*-parasitized Rag-$2^{-/-}$ splenocytes using anti-CD11c coupled magnetic beads before inoculating into WT mice. As shown in Fig. 3(D–F), the outcome of disease by inoculation with DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes was dramatically changed as compared with untreated parasitized cells (Fig. 3D and E, closed circles). As revealed by progressive footpad swelling and high parasite burdens in the footpad as well as in the popliteal lymph node, BALB/c WT mice inoculated with DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes exhibited nearly the same degree of severity as the mice inoculated with DC-depleted parasitized BALB/c-Rag-$2^{-/-}$ splenocytes (Fig. 3D and E, open circles). Notably, both groups of mice were unable to mount T_h1 immune responses (Fig. 3F, open and gray bars). We observed little effect of DC depletion when parasitized splenocytes were inoculated into syngeneic WT mice (e.g. open circles in Fig. 3A and D and open squares in Fig. 3A and D). It is likely that DCs of recipient mice captured *L. major* and elicited immune responses.

To further examine the importance of DCs, DC-depleted parasitized Rag-$2^{-/-}$ splenocytes were reconstituted with parasitized DCs and inoculated into BALB/c WT mice. As shown in Fig. 4, when mice were inoculated with DC-depleted parasitized BALB/c-Rag-$2^{-/-}$ splenocytes together with parasitized B10.D2-DCs, those mice exhibited milder symptoms and induced a T_h1-dominant response (Fig. 4A–C, closed symbols). Such milder symptoms were not observed with parasitized BALB/c-DCs. Moreover, when mice were inoculated with DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes together with parasitized BALB/c-DCs, they exhibited the exacerbated symptoms and resulted in a T_h2-dominant response (Fig. 4D–F, open symbols). Collectively, these results indicate that DCs are indeed the cells that carry genetic factors determining the susceptibility to *L. major* infection. Essentially, the same results were obtained using BMDCs instead of splenic CD11c$^+$ DCs (data not shown).

Discussion

Our present results collectively indicate that DCs carry genetic factors determining the T_h1/T_h2 balance and outcome of *L. major* infection. It is intriguing that macrophages are not involved in determining the T_h1/T_h2 balance. Although macrophages are able to present antigens, macrophages present microbial antigens to differentiated effector cells, especially T_h, to receive cytokines from T_h at the site of infection. On the other hand, DCs activate naive T cells into effector cells in secondary lymphoid organs. Such differences may contribute to the critical role of DCs in determining the T_h1/T_h2 balance.

A previous report has implicated that the genetic difference in susceptibility to *L. major* is determined by both T and

non-T cells by similar experiments using athymic BALB/c recipients reconstituted with T cells from C57BL/6.C-H-2^d congenic mice (20). However, all BALB/c-Rag-$2^{-/-}$ mice reconstituted with B10.D2 T cells exhibited a body weight loss and hair loss and half of these mice died within 3 months (K. Suzue and S. Koyasu, unpublished observation) likely due to the chronic graft versus host reaction by mismatching minor histocompatibility antigens such as minor lymphocyte-stimulating superantigen (21). We were therefore unable to compare the difference between B10.D2-Rag-$2^{-/-}$ mice with BALB/c $CD4^+$ T cells and BALB/c-Rag-$2^{-/-}$ mice with B10.D2 $CD4^+$ T cells.

At the moment, little is known about factors specifically expressed in DCs. Among cytokines produced by DCs, IL-12 is a pivotal cytokine inducing the T_h1 response and is one of likely candidates. However, involvement of IL-12 in the difference between *L. major*-susceptible and -resistant strains is unclear. Previous studies have shown that there is no difference between fetal skin-derived DCs from C57BL/6 and BALB/c in their ability to produce IL-12 in response to *L. major* amastigotes (18, 22). Other studies have also observed little difference in the production of IL-12 by DCs from C57BL/6 and BALB/c mice in response to various stimuli (23). *L. major* infection *in vitro* induced IL-12 release from splenic DCs and splenic DCs from B10.D2 mice produced slightly higher amounts of IL-12 than those from BALB/c mice in our hands (K. Suzue and S. Koyasu, unpublished observation). However, it is unclear if the amount of IL-12 produced by DCs is the only factor determining T_h1/T_h2 balance in *L. major* infection.

It is generally accepted that the T_h1/T_h2 balance in *L. major* infection is a polygenic phenomenon (24). Indeed, in addition to IL-12 handful factors have been reported that affect T_h1/T_h2 balance in a strain-dependent manner. Transforming growth factor-β is known to block T_h1 differentiation at lower doses in BALB/c mice than in other strains of mice by controlling IL-12 receptor expression on T cells (25). IL-1 and tumor necrosis factor-α have been reported as critical factors in BALB/c but not in C57BL/6 mice for the induction of T_h1 response (26, 27). Chemokine/chemokine receptor system is also an important factor that determines the outcome of *L. major* infection (28). These cytokines/chemokines are produced by DCs but by other cell types as well. There was no significant difference in the amounts of IL-1 produced by DCs between B10.D2 and BALB/c mice (K. Suzue and S. Koyasu, unpublished observation). Furthermore, there was no difference in the up-regulation of cell-surface markers including B7 and MHC class II molecules on splenic DCs between these two strains (K. Suzue and S. Koyasu, unpublished observation).

Tpm1 locus is known to control IL-12 responsiveness in a cell-autonomous manner (29). However, *Tmp1* controls IL-12 responsiveness of T cells and our results exclude the involvement of T cells. It was shown that the early administration of IL-4 stimulates DCs to produce IL-12 and protect BALB/c mice from *L. major* infection (30). Such responsiveness of DCs to IL-4 may be important in determining the susceptibility to *L. major* infection. Finally, prostaglandin E_2 (PGE_2) suppresses IL-12p70 production and BALB/c APCs express higher levels of PGE_2 receptors than those of other strains, making BALB/c APCs more sensitive to PGE_2 (31). These possibilities should be examined in future studies.

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Abbreviations

APC	antigen-presenting cell
BM	bone marrow
BMDC	bone marrow-derived dendritic cell
DC	dendritic cell
PGE_2	prostaglandin E_2
WT	wild type

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研究成果の刊行に関する一覧表

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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古西　満，善本英一郎	免疫再構築症候群 呼吸器症候群（第２版）Ｉ ―その他の呼吸器疾患を含めて―	日本臨牀(別冊) 新領域別症候群シリーズ	8	345-348	2008
善本英一郎，古西　満，宇野健司，中川智代，米川真輔，笠原　敬，前田光一，三笠桂一	Tenofovir過量内服を含むHAART開始後短期間に急性腎不全をきたしたHIV感染者の１例	感染症学雑誌	82	650-653	2008

A Conventional LC-MS Method Developed for the Determination of Plasma Raltegravir Concentrations

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Raltegravir belongs to a new class of antiretrovirals acting for a human immunodeficiency virus (HIV)-1 integrase inhibition. Clinical trials of this drug have demonstrated potent antiviral activity in both therapy naïve and experienced patients. Thus, raltegravir has become an important component of combination treatment regimens used to treat patients with multidrug-resistant HIV-1. The quantification of raltegravir in human plasma is important to support clinical studies and determine pharmacokinetic parameters of raltegravir in HIV-1 infected patients. Recently, the LC-MS/MS superfine system was developed to determine plasma concentration of raltegravir; however, the system needs to be delicately set and the equipment is very expensive. Therefore, we developed a conventional LC-MS method to overcome these difficulties. Subsequently the method was validated by estimating the precision and accuracy for inter- and intraday analysis in the concentration range of 0.010—7.680 μg/ml. The calibration curve was linear in this range. Average accuracy ranged from 97.2 to 103.4%. Relative standard deviations of both inter- and intraday assays were less than 10.4%. Recovery of raltegravir was more than 80.6%. This novel LC-MS method is accurate and precise enough to determine raltegravir levels in human plasma samples.

Key words human immunodeficiency virus-1; LC-MS; therapeutic drug monitoring; raltegravir

The clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection has been advanced by the success of highly active antiretroviral therapy (HAART). However, it became clear that the long-term administration of HAART was limited by toxicity associated with many of these treatments[1,2] as well as by the development of resistance.[3—6] Therefore, new antiretroviral drugs, which act on different action points from DNA elongation and protein processing in HIV-1 life cycle, are required to continue effective HAART for the treatment of HIV-1.

Raltegravir is one of a new class of antiretroviral agents that work by inhibiting the insertion of viral DNA into the cellular genome, resulting in virus replication prevention.[7—10] Therefore, raltegravir is expected to treat therapy-experienced patients where protease inhibitor (PI) and/or reverse transcriptase inhibitor-resitant HIV-1 had developed.[11—13]

We have a routine system, by which all PI and efavirenz plasma concentrations are easily determined by HPLC,[14] and therapeutic drug monitoring was performed as needed.[15] In this study, we aimed to develop the determination method of plasma raltegravir.

Recently, a determination method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been reported.[16] However, the MS-MS detector needs to be delicately set and LC-MS/MS equipment is very expensive. In addition, isotope labeled raltegravir as an internal standard (IS) is needed. To bypass these difficulties, we aimed to develop more conventional procedures for determining raltegravir using liquid chromatography coupled with mass spectrometry (LC-MS).

MATERIALS AND METHODS

Chemicals and Reagents Raltegravir was supplied by Merck Research Laboratories (Rahway, NJ, U.S.A.) and the internal standard (IS), A-86093:(5*S*,8*S*,10*S*,11*S*)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl)-1-[(2-1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, was provided by Abbott Laboratories (Abbott Park, IL, U.S.A). Their chemical structures are shown in Fig. 1. Methanol, hexane, methylene chloride, and acetonitrile (Kanto Chemical, Tokyo, Japan) were HPLC grade. Ammonium acetate, EDTA and acetic acid were purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q® system (Millipore Corp., Bedford, MA, U.S.A.). All other chemicals and solvents were of analytical grade.

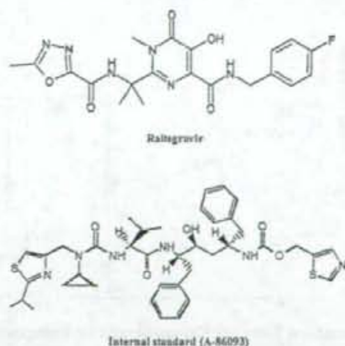

Fig. 1. Chemical Structures of Raltegravir and the Internal Standard A-86093

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Equipment A Waters Alliance 2695 HPLC and a Micromass ZQ-2000 MS (Waters Assoc., Milford, MA, U.S.A.), controlled with MassLynx version 4.0 software, were used for detection. The analytical column was a SunFire C_{18} column (3.5 μm, 2.1×50 mm, Waters), protected by a SunFire C_{18} Guard Column.

Chromatographic and Mass Spectrometric Conditions The mobile phase was a mixture of 0.1 mM EDTA in 0.1% acetic acid (A), 100% acetonitrile (B) and 100% methanol (C). An isocratic mobile phase consisting of A–B–C (65:15:20) was used during the first 2 min of the run, followed by a linear gradient elution consisting of A–B–C (10:70:20) for the next 8 min. The final conditions were maintained for the final 5 min. The system was then reequilibrated for an additional 13 min using the initial conditions. The flow rate of the mobile phase was 0.2 ml/min, the column temperature was 40 °C, and the amount of injected sample was 5 μl.

The mass spectrometer was operated in positive ion electrospray mode. The capillary sprayer voltage was 3.5 kV and the sample cone voltage was 30 V for both raltegravir and A-86093. The source temperature was 120 °C and the desolvation temperature was 350 °C. The desolvation and cone gas flow-rates were set to 600 and 50 l/h, respectively. The acquisition mass range is *m/z* 200—800 at 0.5 s per scan with a 0.1 s interscan delay. All mass spectra are acquired in centroid mode.

Quantitative analysis, carried out in Selected-ion recording (SIR) mode, detected raltegravir at *m/z* 445, and the internal standard (IS), A-86093, at *m/z* 748, all in the form of ions. The quantitation calculations were performed using analytical software, MassLynx version 4.0 (Waters).

Standard Solutions Stock solutions of raltegravir and A-86093 were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50:50, v/v) to yield concentrations of 384.0 μg/ml of raltegravir and 41.0 μg/ml of A-86093. These stock solutions were stored at −80 °C and thawed on the day of analysis. The stock solution was diluted in drug-free plasma to yield raltegravir concentrations of 0.010, 0.192, 1.920, 3.840 and 7.680 μg/ml.

Sample Preparation Two milliliters of methylene chloride/hexane (50:50, v/v) containing the IS (0.328 μg/ml) and 0.3 ml of 0.2 M ammonium acetate were added to a 500 μl plasma sample prepared from peripheral blood anticoagulated with heparin. The mixture was vortexed for 5 min and then centrifuged at 3500 ***g*** for 5 min. The upper layer was separated and evaporated dry. The dried material was then dissolved in 50 μl of a mobile phase solution. Lastly, 5 μl of the upper solution was injected into the LC-MS system. The institutional review board of National Hospital Organization Nagoya Medical Center approved this study and each subject provided written informed consent.

Validation Inter- and intraday precision values using this method were estimated by assaying control plasma containing five different concentrations of raltegravir five times on the same day and on three separate days to obtain the relative standard deviation (RSD). Accuracy was determined as the percentage of the nominal concentration. To assess the absolute recoveries of ralegravir extracted from plasma, the peak area ratios of the analytes to the internal standard were compared with those obtained from the mobile phase having the same concentration. The mean recoveries were determined in triplicate.

RESULTS

LC-MS Chromatograms Figures 2A and B show selected-ion recording chromatograms obtained from a spiked plasma sample containing 0.192 μg/ml of raltegravir and 0.328 μg/ml of A-86093 (IS). Under the described chromatographic conditions, retention times were 8.2 min for raltegravir and 12.9 min for A-86093. Figures 2C and D show chromatograms obtained from a blank plasma sample. Assays performed on drug-free human plasma succeeded to show no interfering peaks during the interested intervals of the retention times. Figure 2D is the expanded figure of the

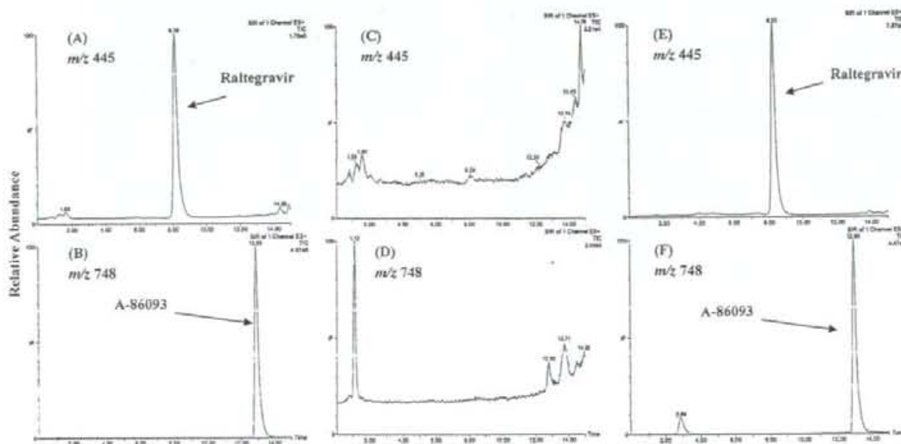

Fig. 2. Selected-ion Recording Chromatograms for Raltegravir and A-86093

(A) and (B) were obtained from a spiked plasma containing 0.192 μg/ml of raltegravir and 0.328 μg/ml of A-86093 (IS). (C) and (D) were obtained from a blank plasma sample. (E) and (F) were obtained from a plasma sample from an HIV-1-infected patient on raltegravir. (A), (C) and (E) were monitored with *m/z* 445. (B), (D) and (F) were monitored with *m/z* 748. (C) and (D) are the expanded figures of the baselines in (A) and (B), respectively.

Table 1. Intraday and Interday Precision and Accuracy for Raltegravir

Expected (μg/ml)	Intraday (n=5)		Interday (n=15)		Accuracy (%)	Recovery (%)
	Measured (μg/ml)	RSD (%)	Measured (μg/ml)	RSD (%)		
0.010	0.010±0.001	8.5	0.010±0.001	10.4	103.4±10.7	83.7±9.8
0.192	0.192±0.011	5.7	0.187±0.016	8.7	97.2±8.5	83.6±3.1
1.920	1.932±0.095	4.9	1.912±0.089	4.6	99.6±4.6	87.4±7.0
3.840	3.872±0.118	3.0	3.825±0.184	4.8	99.6±4.8	80.6±6.5
7.680	7.656±0.097	1.3	7.631±0.186	2.4	99.4±2.4	86.5±6.1

RSD, relative standard deviation. Means±S.D.

Table 2. Plasma Raltegravir Concentrations after the Oral Administration of 400 mg of Raltegravir to an HIV-1-Infected Patient

Time (h)	Raltegravir concentration (μg/ml)
0	1.24±0.03
0.5	1.78±0.02
1	5.21±0.17
2	3.14±0.05
3	2.67±0.04
6	1.46±0.02

Means±S.D.

baseline part of Fig. 2B. These peaks did not affect the quantification of IS. Figures 2E and F show chromatograms of a plasma sample from an HIV-1-infected patient treated with raltegravir. There were no interfering peaks affecting quantification of raltegravir in this chromatogram. Anticoagulants of heparin and EDTA did not hinder the selected-ion recording chromatograms for raltegravir and A-86093.

Validation: Linearity, Precision, Accuracy and Recovery Calibration curves of raltegravir appeared linear in the concentration range of 0.010 to 7.680 μg/ml with a correlation of 1.000.

Precision, accuracy, and recovery of our LC-MS method are shown in Table 1. The selected concentration of raltegravir covers the expected plasma concentrations found in the patients. The RSDs calculated for raltegravir in the inter- and intraday assays ranged from 1.3 to 10.4%, which are similar to previously reported values.[16] Accuracies ranged from 97.2 to 103.4%. Recoveries from plasma ranged from 80.6 to 87.4%. Mean extraction recovery of the IS was 87.9%. These results indicate that this method achieves a high degree of reproducibility and accuracy.

Raltegravir Concentrations in Plasma Plasma raltegravir concentrations in an HIV-1-infected patient are shown in Table 2. The patient received oral administration of 400 mg raltegravir twice daily. The samples were collected on day 8 after the start of HAART. When raltegravir is administered by a single 400 mg dose, plasma concentrations are expected in the 0.01 to 4.71 μg/ml range.[16—18] In this study, raltegravir concentrations at steady state following multiple-dose administration ranged from 1.2 to 5.2 μg/ml.

DISCUSSION

Clinical trials of raltegravir have demonstrated potent antiviral responses in both therapy naïve and experienced patients.[11,19,20] Moreover, raltegravir has demonstrated a clean safety profile in these studies and may not have the toxicity and tolerability issues as the current anti-HIV drugs. Thus, raltegravir has become an important component of combination treatment regimens and its use has been initiated for the treatment of heavily pretreated patients with a multidrug-resistant virus.

We first wanted to judge whether therapeutic drug monitoring of raltegravir is necessary. To achieve this, the development of determination method for raltegravir is essential. Until now there has been a methodological report for the determination of raltegravir using LC-MS/MS.[16] However, this method has several disadvantages in terms of cost performance and essential equipment; for example, the authors used isotoped labeled raltegravir and/or the setting of the LC-MS-MS equipment.

To avoid such disadvantages we decided to use an LC-MS method using an available IS (A-86093) for determining plasma protease inhibitor concentrations. The reason we chose ritonavir analogue A-86093 is the stability and better elution point of the compound on the HPLC as were reported previously.[14] Validation showed our method was successful in measuring plasma raltegravir with high precision and satisfactory RSD values. The raltegravir calibration curve was linear at the concentration range of 0.010 to 7.680 μg/ml, and the average accuracy ranged from 97.2 to 103.4%. Both inter- and intraday RSDs for raltegravir were less than 10.4%, which is similar to previously reported values.[16] Recovery of raltegravir was more than 80.6%. These results indicate our newly developed method achieves the same level of reproducibility and accuracy as the LC-MS/MS method. As plasma concentrations of raltegravir are expected in the 0.01 to 4.71 μg/ml range when raltegravir is administered at single dose of 400 mg,[16—18] our method successfully covers this region with good precision and accuracy. Actually, the raltegravir concentration change was clearly demonstrated; it rose from 1.2 (0 h) to 5.2 μg/ml (1 h), then decreased to 1.5 μg/ml (6 h) when raltegravir was orally administered 400 mg twice daily in an HIV-1-infected patient.

Recently, Poirier *et al.* reported the HPLC method for determining plasma raltegravir concentration with fluorescence detection.[21] Our's and Poirier's methods can specifically determine the raltegravir concentration and the sensitivities seem almost equivalent. Therefore, an alternative use is possible according to the availability of the equipments.

This conventional LC-MS method can provide a routine clinical application, and permits management of drug interactions and toxicity.

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I 感染症呼吸器疾患

I. 感染症関連病態

免疫再構築症候群

Immune reconstitution inflammatory syndrome

Key words：免疫再構築症候群，抗HIV治療

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はじめに

抗HIV(human immunodeficiency virus)治療は進歩し，HIV感染者の予後は劇的に改善している[1]．その一方で，抗HIV治療によって様々な有害事象が生じることも明らかになっている．免疫再構築症候群(immune reconstitution inflammatory syndrome：IRIS)もその一つであり，本稿ではIRISの概説をするとともに呼吸器疾患との関連について紹介する．

1. 概　　念

免疫不全に陥ったHIV感染者に抗HIV治療を開始したときに，日和見感染症などが発症，再発，再増悪することを経験する．これは，抗HIV治療による急速なHIV量減少に伴って単球・マクロファージ・NK細胞などの機能が回復することやCD4陽性細胞が増加することで，患者の免疫能が改善し，体内に存在する病原微生物に対する免疫応答が誘導されるために起こるものと考えられている[2]．このような機序が想定されているため，抗HIV治療開始後に認める日和見感染症などの発症，再発，再増悪はIRISと呼ばれている．

2. 病因(発症リスク)

抗HIV治療を開始する前にIRISの発症リスクを把握できることは有益であり，IRIS発症に関連した臨床的因子が解析されている．French ら[3]は，IRISを起こした症例は起こしていない症例に比べ，抗HIV治療開始時のCD4陽性細胞数が低く(88 vs 237/μL，$p=0.0001$)，HIV-RNA量が高い(5.36 vs 4.88 $\log_{10}$ コピー/mL，$p=0.007$)と報告している．著者らの調査結果[4]も同様で，CD4陽性細胞数が低く(35 vs 128/μL，$p<0.001$)，HIV-RNA量が高い(5.2 vs 4.7 $\log_{10}$ コピー/mL，$p<0.001$)．しかし，抗HIV治療開始時のCD4陽性細胞数やHIV-RNA量には差がないとの報告もあり[5]，まだ一定の見解は出ていない．

Shelburne ら[6]は，IRISを起こした症例は抗HIV治療開始後のHIV-RNA量減少が速やかで，顕著であることを指摘している．著者らの調査結果[4]も同様で，IRISを起こした症例では抗HIV治療開始1カ月後のHIV-RNA量減少が有意に大きい(2.2 vs 2.0 $\log_{10}$ コピー/mL，$p<0.001$)．したがって，抗HIV治療の抗ウイルス効果が良好であることがIRIS発症に関連していると考えられる．

表1には，IRISの発症に関連した臨床的因子の報告内容を示す．今後の詳細な解析が必要ではあるが，**表1**のような因子がある症例に抗HIV治療を始める際には，IRISの発症に注意しながら経過をみる必要がある．

3. 病　　態

IRISの発症頻度は，我が国の調査では抗HIV治療例全体の8.7%で，施設によってその発症頻度に差がある[7]．

IRISとして指摘されている疾患は日和見感染

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表1 免疫再構築症候群の発症に関連した臨床的因子

報告者	臨床的因子	報告誌
French ら	○抗 HIV 治療開始時の CD4⁺数が低値 ○抗 HIV 治療開始時の HIV-RNA 量が高値	HIV Med 1, 2000
Shelburne ら	○男性 ○抗 HIV 治療で HIV-RNA 量が急速に減少 ○日和見感染症診断時に抗 HIV 治療が未実施 ○日和見感染症治療と抗 HIV 治療開始の間隔が短期間	AIDS 19, 2005
Ratnam ら	○より若年 ○抗 HIV 治療開始時の CD4⁺%が低値 ○抗 HIV 治療開始時の CD4/8 比が低値	Clin Infect Dis 42, 2006
Robertson ら	○日和見感染症の既往が多数 ○抗 HIV 治療開始時の CD8⁺数が高値 ○抗 HIV 治療開始時の ALT が低値 ○抗 HIV 治療開始時のヘモグロビンが低値	Clin Infect Dis 42, 2006
古西ら	○抗 HIV 治療開始時の CD4⁺数が低値 ○抗 HIV 治療開始時の HIV-RNA 量が高値 ○抗 HIV 治療 1 カ月後の CD4⁺数・CD8⁺数の増加率が大きい ○抗 HIV 治療 1 カ月後の HIV-RNA 量減少が大きい	「重篤な日和見感染症の早期発見と最適治療に関する研究」平成 19 年度報告書, 2008

症だけでなく，多彩である(**表2**)．我が国で頻度の高い疾患は，帯状疱疹，非結核性抗酸菌症，サイトメガロウイルス(CMV)感染症，ニューモシスチス肺炎，結核症，Kaposi 肉腫などである[8]．以下には，呼吸器疾患の病型を呈することがある IRIS(**表2**では＊)について述べる．

HIV 感染症の非結核性抗酸菌症は，播種型を呈することが多いが，IRIS の病型では播種型，リンパ節炎型，肺感染症型が同頻度で認められる．肺感染症型では自覚症状が軽微なこともあり，胸部画像検査が重要である．

CMV 感染症は，IRIS でも網膜炎などの眼疾患が多い．しかし，腸炎や肝炎，肺炎の病態を示すこともある．肺炎症例では，発熱と呼吸器症状があり，診断の契機となる．

ニューモシスチス肺炎も 38℃以上の発熱があり，呼吸器症状を伴っている．IRIS では気管支肺胞洗浄で *Pneumocystis jirovecii* を証明できる場合とできない場合とがある．血清 β-D グルカン値は上昇することが多く，補助診断に役立つ．

IRIS としての結核症の病型には，肺感染症型，リンパ節炎型，播種型，胸膜炎型がある．培養検査，PCR 法，生検組織診断，ツベルクリン反応を用いて診断する．QuantiFERON-TB の意義については明らかにされていない．

海外では，IRIS としてサルコイドーシスの症例報告がみられている．病状は，非 HIV 感染者のサルコイドーシスと大きな差はなく，縦隔リンパ節腫大や肺野病変の頻度が高い．サルコイドーシス診断時の CD4 陽性細胞数が 200/μL 以上，HIV-RNA 量が低値のことが多い．

4. 診断と鑑別診断

IRIS の診断基準として広く受け入れられているものはないが，**表3**に Shelburne ら[9]が提案した基準を示す．4)の項目が IRIS と鑑別すべき病態であるが，実際には臨床的に鑑別困難なこと

表2 免疫再構築症候群として報告されている疾患
(*は呼吸器病変をきたすことがある疾患)

■抗酸菌	■皮膚疾患
結核症*	疣贅
非結核性抗酸菌症*	好酸球性毛嚢炎
■ヘルペスウイルス	■消化管感染症
サイトメガロウイルス感染症*	虫垂炎
単純ヘルペスウイルス感染症	胆嚢炎
帯状疱疹	膵臓炎
EBウイルス感染症	■Guillain-Barré症候群
Kaposi肉腫*	■自己免疫疾患
■肝炎ウイルス	SLE
B型肝炎	血管炎
C型肝炎	Reiter症候群
■進行性多巣性白質脳症	関節リウマチ
■その他のウイルス	多発性筋炎
パピローマウイルス感染症	Graves病
パルボウイルスB19感染症	全身性脱毛症
BKウイルス感染症	■アレルギー反応
■原虫・真菌感染	刺青の過敏症
トキソプラズマ症	■新生物
ミクロスポリジウム症	Kaposi肉腫*
リーシュマニア症	悪性リンパ腫*
クリプトコッカス症*	非定型リンパ増殖性疾患*
ニューモシスチス肺炎*	■その他
ヒストプラスマ症*	無菌性骨壊死(?)
■その他の感染症	女性化乳房(?)
バルトネラ・ヘンゼレ感染症	サルコイドーシス*
クラミジア・トラコマチス感染症	動脈硬化(?)

(Stoll, et al: Curr Infect Dis Rep 5: 266, 2003. より改変)

表3 免疫再構築症候群に対するShelburneらの診断基準

1) HIV感染陽性
2) HAARTを実施
  ○治療前値よりもHIV-1 RNA量の減少
  ○治療前値よりも$CD4^+$細胞数の増加
3) 炎症反応に矛盾しない症候
4) 臨床経過が以下のことで説明できないこと
  ○既に診断されている日和見感染症の予測される経過
  ○新たに診断された日和見感染症の予測される経過
  ○薬剤の副反応

(Shelburne, et al: J Antimicrob Chemother 57: 167, 2006. より引用)

もありうると考えられる.

現時点では，免疫不全があるHIV感染者に対して新規に抗HIV治療を開始，もしくは効果不十分な治療を変更後，数カ月以内に日和見感染症などの疾患が発症，再発，再増悪した場合にはIRISと考えて対応するのが妥当である．この際も，抗HIV治療の有効性(血中HIV-RNA量低下)を確認することや抗HIV薬などの副作用を除外する必要がある.

5. 治療と予後

IRISを発症した場合も，有効な抗HIV治療をできるかぎり継続することが基本である．IRISへの対処方法には，その疾患自身に対する治療と過剰な炎症のコントロールがある．

疾患が感染症である場合には，病原体の増殖がなければ抗微生物薬の投与は不要とする考え方もある．しかし，臨床的に病原体の増殖がない証明は困難であり，抗微生物薬の開始・追加・変更が必要である．

炎症コントロールの方法には，NSAIDsや副腎皮質ステロイド薬の投与がある．副腎皮質ステロイド薬は，臓器の機能障害が重篤な場合，生命の危機がある場合，他の方法が無効な場合などに考慮する．プレドニゾロン1mg/kg/日で開始し，週から月単位で減量していく方法などが行われている．

IRISのために抗HIV治療を中止することもあるが，その基準も定まっていない．現時点では，抗HIV治療を継続することでIRISの病態が生命を脅かす場合や副腎皮質ホルモン薬が無効な場合などに中止する．

IRISの生命予後は良好なことが多い．しかし，非結核性抗酸菌症，CMV感染症，結核症は病状が安定するのに3カ月以上を要することがしばしばである．特に，非結核性抗酸菌症では副腎皮質ステロイド薬の併用や抗HIV治療の中止が必要となり，難渋することも多い[8]．一方，Parkら[10]は日和見感染症を発症し，かつIRISを発症した症例の長期予後がIRISを発症しなかった症例に比べ，良好であることを報告している．したがって，IRISを単純に抗HIV治療の副作用と位置づけてしまうとIRISの真の病態を見誤る可能性があり，今後の研究成果に注目する必要がある．

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症　例

Tenofovir 過量内服を含む HAART 開始後短期間に急性腎不全をきたした HIV 感染者の1例

奈良県立医科大学感染症センター

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米川　真輔　笠原　敬　前田　光一　三笠　桂一

(平成20年3月17日受付)
(平成20年7月14日受理)

Key words : Tenofovir, human immunodeficiency virus (HIV), renal failure

序　文

新たな抗 HIV (human immunodeficiency virus) 薬が開発され，HAART (highly active antiretroviral therapy) は進化を続けている．抗 HIV 作用が強く，服薬しやすい薬剤が登場し，HIV 感染者の予後はさらに改善している[1]．しかし抗 HIV 薬は副反応が発現しやすく，消化器症状のような各薬剤に共通するものと薬剤ごとに特徴的なものとが存在する．

今回，我々は tenofovir (TDF) を含む HAART 開始時短期間に急性腎不全を合併し，血液透析によって改善した症例を経験した．腎不全発症時の状況や腎生検の所見などが臨床医にとって示唆に富むと考えたので，若干の文献的考察を加えて報告する．

症　例

患者：58歳，男性．

主訴：発熱・嘔気・嘔吐・無尿．

既往歴：48歳時，急性B型肝炎．57歳時，大腸ポリープ．

家族歴：特になし．

生活歴：喫煙歴　なし，飲酒歴　機会飲酒，同性間性的接触歴あり．

現病歴：2005年11月に他院で大腸ポリープの経過観察目的で大腸内視鏡を受け，アメーバ腸炎と診断された．その際に HIV 感染を指摘されたため，2006年1月25日に加療目的で当科を紹介された．初診時の CD4 陽性リンパ球数が 81/μL，HIV-RNA 量が 1.1×10^5 コピー/mL，血清クレアチニン値が 0.8mg/dL で尿蛋白は陰性あった．アメーバ腸炎に対して Metronidazole (2.25g/日) を10日間投与し，途中から血便は消失した．2月に入り鼻水等の感冒様症状が，3月初め頃にも微熱が出現したため市販の総合感冒薬を不定期に服用していた．2月27日に薬剤師による抗 HIV 薬に関する服薬指導を受け，3月16日から TDF + lamivudine (3TC) + lopinavir/ritonavir (LPV/r) による HAART を開始した．その後も発熱があると，消炎鎮痛薬などを併用していた．3月20日に受診した際に血清クレアチニン値が 4.3mg/dL と上昇していたので，薬剤性の腎障害を疑い，全ての抗 HIV 薬を中断した．その後も水分を十分摂取しているにもかかわらず無尿となり嘔気や嘔吐もともなうようになったため，翌日救急外来を受診した．血清クレアチニン値が 8.0mg/dL とさらに上昇していたため，急性腎不全の診断で同日緊急入院となった．

入院時現症：身長 165.0cm，体重 62.0kg，体温 35.9℃，血圧 150/90mmHg，脈拍 72/分・整，呼吸数 16/分・胸腹式．眼瞼結膜に貧血・黄染はなかった．口腔内では舌に白苔を認めた．表在リンパ節は触知しなかった．心音は清で雑音なく，肺音も副雑音を聴取しなかった．腹部は平坦，軟であった．皮膚，四肢，神経系には異常所見を認めなかった．

入院時検査所見 (Table 1)：末梢血検査では，ヘモグロビンが 10.9g/dL，白血球は 11,000/μL と増加し左方移動していた．CRP は 19.6mg/dL と上昇していた．生化学検査ではアルブミン 2.5g/dL と低値，血清学検査では IgA 1,236.8mg/mL，IgE 558.2U/mL と上昇，CD4 陽性リンパ球数が 127/μL と低下，β_2-MG 27.3 mg/L，HIV-RNA 量は 1.8×10^3 コピー/mL であった．ツベルクリン反応は陰性であった．発症直前に使用した薬剤 (Loxoprofen Sodium, Salicylamide, Acetaminophen, Anhydrous caffeine, promethazine Meth-

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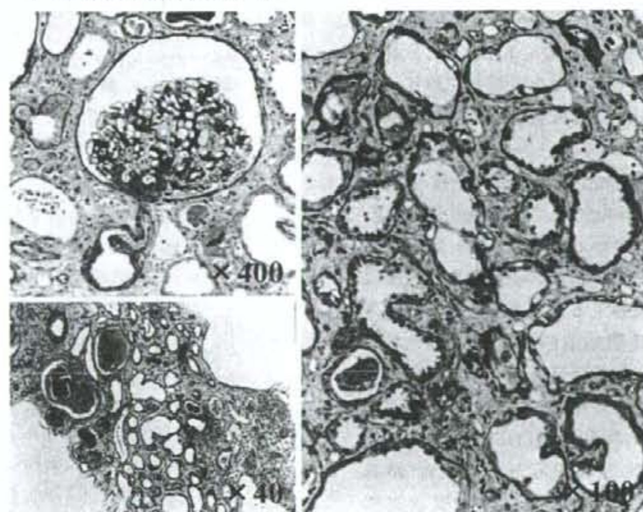
Table 1 Laboratory findings on admission

Peripheral blood		Blood chemistry		Serology/immunology	
RBC	363×10^4 /μL	TP	6.8 g/dL	CRP	19.6 mg/dL
Hb	10.9 g/dL	Alb	2.5 g/dL	β2-MG	27.3 mg/L
Ht	30.6 %	GOT	31 IU/L		
WBC	11,000 /μL	GPT	23 IU/L	IgA	1,236.8 mg/dL
		LDH	370 IU/L	IgG	1,649.1 mg/dL
stab	4 %	ALP	263 IU/L	IgM	54.6 mg/dL
seg	93 %	γ-GTP	62 IU/L	IgE	558.2 U/mL
eos	0 %	BUN	60 mg/dL		
bas	0 %	CRE	8.0 mg/dL	CD4 +	127 /μL
lym	1 %	Na	129 mEq/L	CD8 +	380 /μL
mon	2 %	K	3.8 mEq/L	CD4/CD8	0.32
		Cl	91 mEq/L	HIV-RNA	1.8×10^3 copies/mL
Plt	13.4×10^4 /μL	GLU	172 mg/dL		

Fig. 1 Renal biopsy specimen. (PAS staining)
Some glomeruli are globally sclerotic and the remainder show mild mesangial proliferation. Interstitia are widened and fibrosis exists together with mononuclear cell infiltration. Some tubes are lined by quite flattened epithelial cells and the lumina appear to be dialated. Small arteries show severe intimal thickening and hyalinosis.



ylenedisalicylate）の DLST を行ったが，全て陰性であった．服薬中止 2 日後の TDF 血中濃度は 4,337ng/mL と高値であった．

腎臓超音波検査：のう胞を認めたが，両腎の萎縮はなかった．実質は高エコーだったが，血流信号は異常を認めなかった．

腎生検標本（Fig. 1）：メサンギウムの軽度増殖や細動脈の硬化，糸球体の硬化，間質の繊維化が見られた．さらに尿細管上皮の消失や空包化など尿細管壊死像も認めた．

入院後経過：薬剤性急性腎不全を疑い，全ての薬剤を中止した．翌日より 4 日連続して血液透析を施行し，以後週 3 回の透析を行った．徐々に尿量は回復し，入院 2 週間目頃からは尿量も確保できるようになった．腎機能も回復してきたため 4 月 15 日に血液透析を終了した．血清クレアチニン値が 3.0mg/dL 台で病状が安定したため，5 月 17 日に退院した．入院後に TDF を誤って 1 日 2 回，つまり倍量服用していたことが判明した．

考　察

核酸系逆転写酵素阻害薬である TDF は，その強力な抗 HIV 作用と 1 日 1 回服用という投与の簡便さから様々なガイドラインにおいて初回治療の推奨される組み合わせに含まれており[2]，処方の機会が増えている．HIV 感染者における腎障害には，巣状分節性糸球体硬化症を伴う狭義の HIV 関連腎症や HIV 関連免

平成20年11月20日

疫複合性増殖性腎炎・HIV 関連血栓性微小血管障害などが報告されている．一方で，抗 HIV 治療や日和見疾患治療のための薬剤による腎障害も報告され[3]，本邦でも TDF に関連した腎障害の報告例が散見されている[4~7]．Nelson ら[8]によると，市販後4年間の調査では TDF による重篤な腎障害の発現率は 0.5% と報告され，その危険因子として腎毒性を有する薬剤との併用・免疫不全が進行した時期・腎疾患の既往・敗血症の合併を挙げている．

本症例は，HIV 感染を家族に知られたくないために服薬指導の際に使用した説明用紙や処方したボトルの薬品名ラベルを捨てており，記憶に頼って服薬していた．その結果，TDF を誤って1日2回，倍量の 600 mg/日で服用していた．I/II 相試験[9]では，TDF の血中濃度は投与量が 75~600mg/日の範囲で用量に比例し，トラフ値は 300mg/日で 50ng/mL，600mg/日で 100ng/mL であったが有効性・安全性に差はないとされていた．本症例では投与中止2日後にもかかわらず血中濃度は 4,337ng/mL と異常高値を示していた．このことは，腎障害が高度になると，TDF の血中濃度は異常高値を長時間持続することを示唆しており，この血中濃度の高値がさらなる腎障害を惹起する可能性が推察された．

I/II 相試験において 600mg/日の TDF を投与された症例でのトラフ値は 100ng/mL で 300mg/日投与の症例と比し安全性に差はないとの結果から，誤って倍量の TDF を服用したことだけで本症例における腎障害の発症原因をすべて説明することができないと考える．本症例は，HAART 開始前の血清クレアチニン値が正常であったが，腎生検組織所見で糸球体の硬化像などが存在し，潜在的な腎障害があった可能性が考えられる．また，LPV/r の併用は TDF の体内動態に影響を与え AUC が 32% 上昇するという報告[10]がある．Whelton ら[10]によると，NSAIDs はプロスタグランジンの産生を抑制する薬理作用をもつ．しかしプロスタグランジンは腎血管拡張作用をもつため，その抑制は腎血流量を減少させ腎前性腎不全を誘発する可能性がある．さらに NSAIDs はアレルギー性の機序による尿細管の間質性腎炎などを引き起こし腎機能に影響の及ぼす可能性が指摘されている．

したがって，本症例では動脈硬化に伴う潜在的な腎障害がある上に TDF を過量に服用し，LPV/r や NSAIDs を併用したことが複合的に作用して腎不全に陥ったと推察する．

様々な事情を抱える HIV 感染患者は十分に服薬指導を行っても服薬方法や服薬量を間違えたり，さらに高齢者では潜在的な腎障害を合併している可能性もあり，その他様々な要因が絡み合って急性腎不全を発症する危険性のあるため，TDF 投与の際には十分な注意が必要であると考える．

なお，本論文要旨は，第20回日本エイズ学会（2006年12月，東京）にて発表した．

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A Case of Acute Renal Failure Involving High Amounts of Tenofovir After HAART Start

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A 58-year-old man admitted for fever, nausea, vomiting, and anuria after the start of HAART, including tenofovir, had a viral load of 1.1×10^5copies/mL, a CD4-positive lymphocyte count of 81/μL, and serum creatinine of 0.8mg/dL before HAART. He underwent renal biopsy and temporary dialysis.

We concluded that the patient had acute tubular necrosis because of potentially impaired renal function and the high amount of medication, and judging from the renal biopsy specimen and clinical course.

When implementing HAART, physicians should be aware of and monitor potential patient misunderstanding of instructions on dosage and administration and for possible complications in medicinal combinations and potential side effects.

TDF taken together with lopinavir may increase the plasma concentration of TDF or other medications that could worsen renal function. It should also be noted that renal dysfunction is a potential complication in the elderly.

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研究成果の刊行に関する一覧表

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雑誌

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An amino acid substitution in PBP-3 in *Haemophilus influenzae* associate with the invasion to bronchial Q1 epithelial cells

Tadashi Okabe[a], Yoshitaka Yamazaki[a,b,*], Miho Shiotani[a], Takefumi Suzuki[a], Mayumi Shiohara[a], Eriko Kasuga[a], Shigeyuki Notake[c], Hideji Yanagisawa[c]

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KEYWORDS
Non-typeable *Haemophilus influenzae*;
fts-I;
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Summary

Haemophilus influenzae is a common pathogen of respiratory infections. We examined whether β-lactamase-negative ampicillin-resistant (BLNAR) strains that are known to have ampicillin resistance due to a substitution of amino acid of penicillin binding protein (PBP)-3, differ from β-lactamase-negative ampicillin-susceptible strains with regard to invasion of bronchial epithelium. After 3 h incubation of each of 32 β-lactamase-negative ampicillin-susceptible and 59 BLNAR strains in the presence of BEAS-2B cells, a human bronchial epithelium cell line, extracellular bacteria were killed using gentamicin and intracellular bacteria numbered. All nine strains in which the efficiency of invasion was 1% or higher were BLNAR strains. The rate of invasion was significantly greater in strains with PBP-3 amino acid substitution (Met377 to Ile, Ser385 to Thr, Leu389 to Phe, and Asn526 to Lys) ($n = 34$) than in those with no amino acid substitution. Electron microscopy showed that high invasive BLNAR strains were observed in cytoplasm of BEAS-2B cell layer. The injured cells were $9.44 \pm 1.76\%$ among attaching cells examined by trypan blue staining after 6 h. These data may suggest that the amino acid substitution of the PBP in BLNAR strains may at least partly play roles in macropinocytosis, leading to the invasion and injury to epithelial cells.
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Abbreviation: PBP: penicillin binding protein, BLNAR: β-lactamase-negative ampicillin-resistant.

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Introduction

Haemophilus influenzae is a pathogenic bacteria responsible for diseases including otitis media, sinusitis, bronchitis, and pneumonia (Bartlett et al. 2000; Pfaller et al. 2001; Ehrlich et al. 2002). It is also known to repeatedly cause airway infection in patients with underlying diseases such as chronic obstructive pulmonary diseases (COPD) and cystic fibrosis (Murphy 2006). Most of the *H. influenzae* strains isolated and identified as pathogenic bacteria for these respiratory infections are non-typeable *H. influenzae* without a capsule in their Q2 outer layer (Murphy and Apicella 1987; Sethi et al. 2002; Bouchet et al. 2003).

Recently, the appearance of strains of *H. influenzae* resistant to β-lactam antimicrobial agents has emerged as a clinical problem. *H. influenzae* acquires resistance to β-lactam antimicrobial agents by either of two mechanisms. One is ampicillin resistance caused by the production of β-lactamases such as TEM-1 and Rob-1 and is called β-lactamase-positive ampicillin-resistant, the other is resistance acquired with a decrease in affinity to β-lactam antimicrobial agents due to amino acid substitution of penicillin binding protein (PBP)-3.

Strains are described here as β-lactamase-negative ampicillin-resistant (BLNAR) (Parr and Bryan 1984; Mendelman et al. 1990; Ubukata et al. 2001; Dabernat et al. 2002). Although BLNAR is defined by the Clinical and Laboratory Standards Institute/the National Committee for Clinical Laboratory Standards (CLSI/NCCLS) as *H. influenzae* showing with a minimum inhibitory concentration (MIC) of ampicillin of 4 μg/mL or higher, most strains with an ampicillin-MIC of 1 or 2 μg/mL were recently found by *ftsI* sequencing of the PBP-3 gene to have the same PBP-3 amino acid substitution as genetically BLNAR strains (Ubukata et al. 2001). Surveillance in Japan and Spain reported that BLNAR accounted for a high percentage of ampicillin-resistant *H. influenzae*: the genetic ratio of BLNAR was 52.0% (Sanbongi et al. 2006) and 65.1% (Hotomi et al. 2007) in Japan and 56% in Spain (García-Cobos et al. 2007). This trend differs from that in North America, where BLNAR accounts for less than 5%, while β-lactamase-positive ampicillin-resistant Q3 strains are dominant (Karlowsky et al. 2002, Fluit et al. 2005).

Recently, clarification about the mechanism of airway epithelium invasion by *H. influenzae* has been found to internalize in adenoid cells of children (Forsgren et al. 1994). Ketterer et al. (1999) observed that *H. influenzae* invasion of bronchial epithelial cells begins with extension of host cell microvilli and the formation of lamellipodia. Microvilli appear as the cytoskeleton is rearranged by polymerization of 20–30 actin filaments (Holmes and Bakaletz 1997; Ketterer et al. 1999). Q4 During macropinocytosis membrane folds extending from the surface fuse back with the plasma membrane.

Pathogenic bacteria of respiratory infections are mostly non-typeable *H. influenzae* (Murphy and Apicella 1987). Capsular polysaccharide, lipopolysaccharide (LPS), and various other polysaccharides are present in the surface layer of *H. influenzae* (Gotschlich et al. 1981; Kuo et al. 1985). Capsular polysaccharide is classified into six serotypes, a–f, based on the antigenicity. The surface structure of non-typeable non-capsulated strains is composed of outer membrane proteins, such as lipooligosaccharide (LOS), pili, outer membrane proteins (Flesher and Insel 1978; Weiser et al. 1990), and these are considered to be an important etiological factor related to the uptake of non-typeable *H. influenzae* by bronchial epithelial cells. Swords and colleagues showed glycoform containing phosphorylcholine to be of special importance for the invasion of bronchial epithelial cells among LOS of non-typeable *H. influenzae* (Swords et al. 2000). Moreover, its phosphorylcholine activates pertussis toxin-sensitive heterometric G protein complex by binding with platelet activating factor (PAF) receptors of bronchial epithelial cells and induces actin polymerization by putting the cell signal cascade into action (Swords et al. 2000; Wang et al. 2003).

The objective of this study was to investigate whether clinical isolates and the BLNAR strain of *H. influenzae* invade cultured bronchial epithelial cells, and clarify characteristics involved in the invasiveness. In addition, the influence of the BLNAR strain on the invaded bronchial epithelial cell injury was investigated.

Methods and materials

Clinical isolates

Ninety-one clinical isolates of *H. influenzae* were obtained at Shinshu University Hospital and Miroku Medical Laboratory Co. between January 2001 and March 2005 and stored by freezing at −78 °C in a MicroBank (Sanko Junyaku Co., Tokyo). All strains were cultured using chocolate II agar (Japan Becton Dickinson Co., Tokyo) at 35 °C in 5% CO_2 for 15–18 h.

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