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Original Article

Seroprevalence of *Entamoeba histolytica* Infection in Female Outpatients at a Sexually Transmitted Disease Sentinel Clinic in Tokyo, Japan

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SUMMARY: From 2003 to 2006, we surveyed the seroprevalence of amoebic infection in female outpatients at a gynecologist's office, which was designated as a sexually transmitted disease sentinel clinic by the Tokyo Metropolitan Government, using an enzyme-linked immunosorbent assay (ELISA). The annual rate of anti-*Entamoeba histolytica* (HM-1:IMSScl6 strain; HM-1) antibody-positive cases as detected by ELISA increased during that period, and anti-*Chlamydia trachomatis* antibodies were detected in 60%, i.e., 24 of 40 anti-HM-1 antibody-positive individuals, suggesting sexual transmission of *E. histolytica*. We designed an ELISA with better sensitivity using the antigen extracted from the virulence-augmented *E. histolytica* strains (LHM-1 and LLA526 strains) by liver-passaging in hamsters. The average ratios of the S/N value (optical density [OD] of sample/OD of negative control) of ELISA with either the LHM-1 or LLA526 antigen and that of ELISA with the HM-1 antigen were significantly higher in intestinal amoebiasis cases with low S/N values than in amoebic liver abscess cases. In the present study of the seroprevalence of *E. histolytica* infection, the sera testing positive with low S/N values (<10) by ELISA with HM-1 antigen exhibited higher S/N values by ELISA using LHM-1 and LLA526 antigens. This modification of the antigen preparation for ELISA is expected to be effective in detecting anti-*E. histolytica* antibodies from such asymptomatic patients who have low antibody titers.

INTRODUCTION

In Japan, it was thought until the mid-1970s that amoebiasis was solely food borne and spread via food contaminated with cysts of *Entamoeba histolytica*. However, in the late 1970s, after amoebiasis was reported to have spread among men having sex with men (MSM) in large cities of the United States, it was recognized as a sexually transmitted disease (STD) (1,2). Within a few years, the suspected number of MSM having anti-*E. histolytica* antibodies along with anti-*Treponema pallidum* antibodies began to increase in densely populated cities in Japan (3,4).

In data provided by Japan's National Epidemiological Surveillance of Infectious Diseases, the number of notified cases with amoebiasis has been increasing annually; in 2006, 747 cases were reported, approximately 90% of which were male. However, with the spread of amoebiasis, the number of notified female cases has also increased at a slow but steady pace since 1999 (5,6).

In the present study, by detecting anti-*E. histolytica* (HM-1:IMSScl6 strain; HM-1) antibodies using an enzyme-linked immunosorbent assay (ELISA), we report the seroprevalence of amoebic infection in female outpatients who visited a gynecologist's office in Tokyo, Japan, from 2003 to 2006; this office was designated as an STD sentinel clinic by the Tokyo Metropolitan Government.

Moreover, in this study we attempted to design an ELISA with better sensitivity. This involved the use of the antigen extracted from the virulence-augmented *E. histolytica* strains by liver-passaging in hamsters. The serum anti-*E. histolytica* antibody titers are low in a majority of asymptomatic amoebiasis cases. Practically, this serological method using LHM-1 and LLA526 antigens was tested on the anti-HM-1 antibody-positive sera in the present surveillance study.

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MATERIALS AND METHODS

Study population: This study was conducted at a Tokyo gynecologist's office that was designated as an STD sentinel clinic by the Tokyo Metropolitan Government. We collected blood samples from 981 female outpatients between 2003 and 2006 (205 in 2003, 217 in 2004, 282 in 2005, and 277 in 2006) (Table 1). All individuals provided informed consent. Patient age was the only additional information. The anti-*E. histolytica* antibody-positive sera were examined for anti-*Chlamydia trachomatis* and anti-*T. pallidum* antibodies as indicators of STDs.

ELISA: *E. histolytica* antigens were prepared from axeni-

Table 1. Study samples in age categories from 2003 to 2006

Age	2003	2004	2005	2006	Total
<20	15	18	22	12	67
20-24	53	56	71	79	259
25-29	69	59	83	90	301
30-34	41	54	57	41	193
35-39	10	18	25	24	77
40-44	8	4	8	12	32
45-49	2	1	8	1	12
50<	1	2	5	7	15
unknown	6	5	3	11	25
Total	205	217	282	277	981

cally cultured *E. histolytica* (HM-1: ATCC no. 50527). The antigen was diluted with 0.05 M bicarbonate buffer to yield a concentration of 5 μg/mL. The diluted antigen (100 μL) was pipetted into each well of the microplate (Nunc-Immuno Module; Nunc Co., Roskilde, Denmark; Cat. no. 469078) and sensitized by incubation for 2 h at 37°C (7). After washing with a buffer (0.15 M phosphate buffer [PB] containing 0.05% Tween 20, pH 7.2; PB/T), 100 μL of the serum samples diluted 1:200 with a dilution buffer (PB/T containing 1% bovine serum albumin [BSA]) were pipetted into the microwells followed by incubation for 40 min at 37°C. The microplate was washed 3 times with PB/T after incubation, and 100 μL of 1:8,000 diluted peroxidase-conjugated anti-human IgG rabbit serum (ICN-Cappel Inc., Aurora, Ohio, USA; Cat. no. 55221) was added, followed by incubation for 40 min at 37°C. After washing with PB/T, the substrate solution comprising 0.03% 2,2′-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma Co., St. Louis, Mo., USA; Cat. no. A1888), 0.01% H_2O_2 in 10 mL of 0.1 M Na_2HPO_4, and 10 mL of 0.1 M citric acid was added to each well. After 7 min, 50 μL of 1.25% NaF solution was added to arrest color development, and an ELISA reader (MTP-120) (Corona Electric Co., Ltd., Ibaragi, Japan) was used to measure the absorbance at 405 nm. The cut-off S/N optical density (OD) value, calculated using the average OD of negative sera from 5 healthy individuals, was set at 3.

Serological test for *C. trachomatis* and *T. pallidum* infections: Anti-*C. trachomatis* IgG and IgA antibodies were measured using a solid-phase enzyme immunoassay kit (Peptide-Chlamydia IgG and IgA; Ani Labsystems Ltd., Oy, Vantaa, Finland).

Nontreponemal anti-cardiolipin (CL) antibodies were detected using two kits (biologic false-positive tests for syphilis), i.e., slide test antigen (DS Pharma-Biomedicals, Osaka, Japan) and rapid plasma reagin (RPR) test (Sanko Junyaku, Tokyo, Japan). The sera that tested positive by these kits were retested using the *T. pallidum* passive hemagglutination (TPHA) kit (Fujirebio, Inc., Tokyo, Japan) for detection of anti-*T. pallidum* antibody. The OD values of the positive and negative control sera for quality control and the OD value of the positive control of the kits were measured during each run of all the serological tests.

ELISA with antigens from virulence-augmented amoebae: Based on the hypothesis that the amount of antigenic substances would also decrease simultaneously with the loss of virulence, we attempted to design an ELISA with better sensitivity in the following manner: (i) HM-1 and LA526 strains cultured axenically for 3 days in the TYI-S-33 medium were inoculated (dose, 1×10^6 amoebae/0.1 mL/head) into the left hepatic lobes of female Syrian golden hamsters (age, 3-4 weeks) (8). (ii) On the 6th day of inoculation, the hamsters were sacrificed and the livers dissected aseptically. The amoebic abscesses isolated from each of the livers were minced finely and crushed using scissors for medical use in the TYI-S-33 medium (9). (iii) After removing the tissue debris from the amoebic cell suspensions and washing twice in the TYI-S-33 medium by centrifugation (175 × *g* for 3 min), both *E. histolytica* strains were cultured axenically in the TYI-S-33 medium. They were named LHM-1 and LLA526. The long-term axenically cultured HM-1 was passaged 16 times through hamster liver due to the significant reduction in virulence, whereas the LA526 was passaged only once because it was newly isolated from the pus of a human amoebic liver abscess only 8 months earlier. (iv) LHM-1 and LLA526 were mass cultured within 2 weeks after their transfer into TYI-S-33 medium from the amoebic liver abscesses. The antigens were then harvested and washed twice in phosphate buffered saline (PBS) by centrifugation (175 × *g* for 3 min) and suspended in 5 mL of distilled water, followed by intermittent sonication (UH-150; SMT Co., Ltd., Tokyo, Japan) at 10 kHz for 5 min in an ice bath. (v) The sonicated suspensions were then centrifuged at 9,100 × *g* for 30 min, and the protein concentrations of the aqueous soluble extracts were measured by Bradford's method (10). (vi) LHM-1 and LLA526 antigens were sensitized at a concentration of 0.5 μg/well according to the procedures described above.

Each serum sample was tested in triplicate for each of the three antigens–LHM-1, LLA526, and HM-1–and the average OD values were calculated. The sensitivity of ELISA for each of the three antigens was compared with the positive serum samples of 5 patients clinically diagnosed having amoebic liver abscesses and 5 mentally handicapped persons in a rehabilitation institution for the intellectually impaired in Japan, who were almost free from amoebiasis symptoms but positive for *E. histolytica* cysts on microscopy and for *E. histolytica* antigen when tested by using an *E. histolytica*-specific antigen detection kit (*E. histolytica* II kit; TechLab, Blacksburg, Va., USA). In each of the 10 human serum samples obtained as described above from the cases of amoebic liver abscess and asymptomatic cyst passers, the ratio was determined between the S/N values (OD value of serum sample [S]/average OD of negative sera from 5 healthy individuals [N]) of ELISA with the LHM-1 and HM-1 antigens and that between the S/N values of ELISA with the LLA526 and HM-1 antigens.

RESULTS

Seroprevalence of anti-*E. histolytica* antibodies in the female population: During the 4 years 2003 to 2006, in the 981 sera samples obtained from the study population, the seroprevalence of anti-*E. histolytica* (HM-1) antibodies increased every year. In 2005 and 2006, the annual positive rate was >5%; the average annual positive rate over the 4 years was 4.1% (40/981) (Table 2). In addition, 60%, i.e., 24/40 of these cases, were also positive for anti-*C. trachomatis* antibodies–an indicator of STDs. On the other hand, none of the cases were positive for anti-CL antibodies (a retest by the TPHA kit was not performed). The strong positive correlation between seropositivity for anti-*E. hitolytica* and anti-*C. tracomatis* antibodies suggested sexual transmission of *E. histolyrica* in the female population. The age range with the highest number of individuals positive for anti-*E. histolytica* antibodies was that of 25-29 years, with 11, and that of 30-

Table 2. Seroprevalence of anti-*Entamoeba histolytica* antibodies in the female outpatients from a gynecologist's office, Tokyo, Japan, by enzyme-linked immunosorbent assay from 2003 to 2006

Year	No. of samples	No. of positives	Positive rate %	No. of positives for anti-CT antibodies[1]
2003	205	3	1.5	2
2004	217	8	3.7	6
2005	282	14	5.0	7
2006	277	15	5.4	9
Total	981	40	4.1	24

[1]: Number of positives for anti-*Chlamydia trachomatis* (CT) antibodies that were also positive for anti-*E. histolytica* antibodies.

Table 3. Age distribution of the female outpatients from a gynecologist's office with positive for anti-*Entamoeba histolytica* antibodies

Year	20-24	25-29	30-34	35-39	40-44	45-49	50<
2003	1	1 (1)		1 (1)			
2004		2 (1)	3 (3)	2 (2)			
2005	1	2 (1)	3 (1)		3	4 (3)	2 (2)
2006	3 (2)	6 (5)	3 (2)		2		1
Total	5 (2)	11 (8)	9 (6)	3 (3)	5	4 (3)	3 (2)

Number of positives for anti-*Chlamydia trachomatis* antibodies that were also positive for anti-*E. histolytica* antibodies are provided in parentheses.

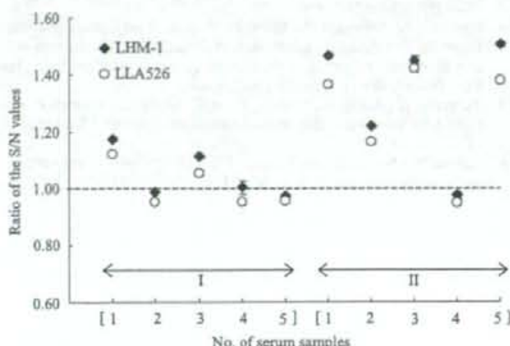

Fig. 1. Ratio of the S/N values of ELISA with better sensitivity performed using the antigens from hamster liver-passaged LHM-1 and LLA526 strains to the S/N values of ELISA using the antigen from the HM-1:IMSScl6 (HM-1) strain in the clinical serum samples of amoebiasis (S/N values: OD of serum sample [S]/average OD of negative sera [N]). The mean of the ratios of triplicate ELISA are plotted. I: Samples ($n = 5$); anti-HM-1 antibody-positive sera from the clinical patients of amoebic liver abscess. The S/N values of ELISA with LHM-1 and LLA526 antigens did not increase significantly ($P > 0.05$ by t test). II: Samples ($n = 5$); anti-HM-1 antibody-positive sera from the mentally handicapped individuals admitted to a rehabilitation institution for the intellectually impaired in Japan. The S/N values of ELISA with LHM-1 and LLA526 antigens increased significantly ($P < 0.05$ by t test).

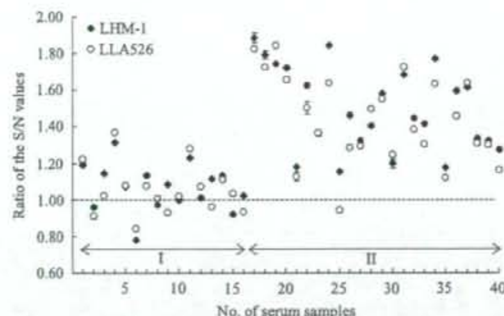

Fig. 2. Ratio of S/N values of ELISA performed using antigens from the LHM-1 and LLA526 strains to the S/N values of conventional ELISA performed using the antigen from the HM-1 strain in female outpatients from a gynecologist's office. The mean of the ratios of triplicate ELISA are plotted. I: Samples ($n = 16$); anti-HM-1 antibody-positive sera with high S/N values (≥ 10). The S/N values of ELISA with LHM-1 and LLA526 antigens did not increase significantly ($P > 0.05$ by t test). II: Samples ($n = 24$); anti-HM-1 antibody-positive sera with low S/N values (<10). The S/N values by ELISA with LHM-1 and LLA526 antigens increased significantly ($P < 0.01$ by t test).

34 years, with 9. The number of individuals positive for anti-*C. tracomatis* antibodies correlated with the number of those positive for anti-*E. hitolytica* antibodies (Table 3).

Comparison of S/N values of ELISA with three different antigens: Figure 1 shows the results of a pilot study in which ELISA with LHM-1 and LLA526 antigens was conducted using sera from 5 human cases each of amoebic liver abscess and asymptomatic cyst passers. The average ratio (1.324) between S/N values of ELISA with the LHM-1 and HM-1 antigens and that (1.254) between S/N values of ELISA with the LLA526 and HM-1 antigens increased significantly only in asymptomatic cases with low S/N values ($P < 0.05$ by t test) and not in amoebic liver abscess cases (1.048 and 1.006, respectively; $P > 0.05$ by t test).

The 40 anti-HM-1 antibody-positive sera as detected by ELISA were classified into two groups based on the magnitude of the S/N values (i.e., groups I and II with S/N values ≥ 10 and <10, respectively). The tendency of ELISA with LHM-1 and LLA526 antigens to yield significantly higher S/N values ($P < 0.01$ by t test) was also confirmed in seropositive cases from among the present study population with low S/N values (<10) by ELISA using the HM-1 antigen (Figure 2).

DISCUSSION

In Japan, the MSM population is still thought to be a major high-risk group for STDs. However, our study provided evidence indicating that the seroprevalence of the *E. histolytica* infection in the female population of Tokyo is increasing annually.

In addition, the result that 60% of the female study population who were anti-*E. histolytica* antibody-positive were also positive for anti-*C. tracomatis* antibodies, an indicator of STD, along with the diversity of sexual behavior suggested that a major proportion of females positive for anti-*E. histolytica* antibodies may have been infected with *E. histolytica* by sexual transmission. We do not fully understand why none of the cases were positive for anti-CL antibodies in the female population, unlike the case in the MSM population (11,12). We are currently conducting further epidemiological studies on the route of *E. histolytica* infection in the female population.

The tendency of ELISA using the LHM-1 and LLA526 antigens to yield statistically higher S/N values ($P < 0.01$ by t test) was evident only in the positive cases with low S/N values (<10) among the present female study population. The active antigenic substance that brought about this effect could not be identified in the present study. Despite the necessity of further evaluation, the improved ELISA is expected to be effective for detecting anti-*E. histolytica* antibodies from such asymptomatic patients who have low antibody titers. Moreover, the hamster liver-passaged *E. histolytica* may be applied as a sensitive antigen to other serodiagnostic methods, such as dot-ELISA (13) and immunofluorescence antibody tests (14).

Because of the public's indifference to STDs, the control of amoebiasis should start with efforts to raise public awareness of the risk of infection by sexual transmission Also simpler and more sensitive mass examination methods should be developed, such as the newly designed ELISA using the antigen extracted from the virulence-augmented *E. histolytica* strains, which have a better sensitivity for the diagnosis of amoebiasis.

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Critical role of dendritic cells in determining the T_h1/T_h2 balance upon *Leishmania major* infection

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Keywords: antigen-presenting cell, infectious disease, protozoan parasite, T_h

Abstract

The onset of T_h1 immunity is in part regulated by genetic background. To elucidate the cell type carrying critical factors determining the T_h1 response, we employed Rag-$2^{-/-}$ mice on *Leishmania major*-susceptible BALB/c and -resistant B10.D2 backgrounds. By using bone marrow (BM) chimeras generated by the transplantation of B10.D2 BM cells into BALB/c-Rag-$2^{-/-}$ mice, and *vice versa*, it was shown that hematopoietic cells carry factors determining the disease outcome and T_h1 response against *L. major* infection. B10.D2-Rag-$2^{-/-}$ mice reconstituted with BALB/c $CD4^+$ T cells exhibited a T_h1 response and controlled *L. major* infection. Wild-type BALB/c mice inoculated with *L. major*-parasitized B10.D2-Rag-$2^{-/-}$ splenocytes also exhibited a T_h1 response and a mild disease outcome, whereas such a T_h1 response was not induced when $CD11c^+$ dendritic cells (DCs) were depleted from parasitized B10.D2-Rag-$2^{-/-}$ splenocytes. T_h1 response was reconstituted by the addition of *L. major*-parasitized B10.D2 DCs but not *L. major*-parasitized BALB/c DCs to DC-depleted parasitized B10.D2-Rag-$2^{-/-}$ splenocytes. These results indicate that DCs determine the outcome of the disease upon *L. major* infection.

Introduction

The balance between two distinct $CD4^+$ T_h responses, namely T_h1 and T_h2 immune responses, is tightly correlated with the outcome of various diseases including tumor, autoimmune diseases and infectious diseases (1). Understanding the basis of the genetic control of T_h1/T_h2 differentiation is important for the development of vaccines and therapeutic strategies. The infection of mice with *Leishmania major*, a macrophage-tropic intracellular protozoan parasite, is widely employed as a model for the functional analyses of T_h1 and T_h2 responses upon microbial infection (2). After infection with *L. major*, most strains of wild-type (WT) mice exhibit a T_h1 immune response that is essential in controlling the intracellular micro-organism. In contrast, BALB/c mice exhibit a T_h2 response and succumb to *L. major* infection (2–4).

Previous studies have reported that a fraction of $CD4^+$ T cells recognizing a specific *Leishmania* antigen produce a large amount of IL-4 during the early phase of infection in naive BALB/c mice (5, 6). Such IL-4 production is considered to be the exacerbation factor of *L. major* infection by instructing naive T cells to elicit a T_h2 response in BALB/c mice (6–8). On the other hand, other reports have shown that B10.D2 mice also produce a large amount of IL-4 but are able to control *L. major* infection and BALB.B mice sharing the same MHC with C57BL/6 and C57BL/10 mice produce only a small amount of IL-4 yet succumb to infection (9, 10). In addition, BALB/c mice mount a T_h2 response upon infection with *L. major* lacking an immunodominant epitope that induces early IL-4 production from $CD4^+$ T cells (11). Furthermore, IL-4-deficient mice on a BALB/c background are still susceptible to *L. major* (12). These findings suggest that cells other than the specific subset of T cells producing IL-4 are crucial in determining the outcome of *L. major* infection in BALB/c mice.

To this end, we performed a series of experiments to determine the cell type carrying the genetic factors that

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controls the outcome of the disease upon *L. major* infection by using Rag-$2^{-/-}$ mice on *L. major*-susceptible BALB/c and -resistant B10.D2 backgrounds. Our results demonstrate that dendritic cells (DCs) play a critical role in determining the T_h1/T_h2 balance as well as the outcome of disease upon *L. major* infection.

Methods

Mice

WT BALB/c and B10.D2 mice were purchased from Japan SLC (Shizuoka, Japan) and Rag-$2^{-/-}$ mice on BALB/c (N12) and B10.D2 (N10) backgrounds (13, 14) were obtained from Taconic (Germantown, NY, USA). Mice were maintained in our specific pathogen-free facility and all experiments were performed in accordance with our Institutional Guidelines.

Antibodies

FITC-, PE- and biotin-labeled mAbs were purchased from BD PharMingen (San Diego, CA, USA). FITC–F4/80 and Red670–streptavidin were purchased from Caltag Laboratories (Burlingame, CA, USA) and GIBCO BRL (Grand Island, NY, USA), respectively. All magnetic bead-conjugated mAbs were purchased from Miltenyi Biotech (Sunnyvale, CA, USA).

Leishmania major *infection*

Leishmania major (MHOM/SU/73/5-ASKH) was maintained in BALB/c mice. Before experiments, parasites were obtained from the infection site on a left hind footpad and promastigotes were propagated at 26°C in Schneider's *Drosophila* medium (GIBCO BRL) containing 15% heat-inactivated FCS. Mice were subcutaneously injected with 5×10^6 of stationary-phase promastigotes at left hind footpads. In some experiments, mice were injected with 1×10^7 of promastigote-parasitized splenocytes from Rag-$2^{-/-}$ mice.

The severity of the disease was evaluated by the footpad swelling and parasite burdens in the infected footpads and, in some experiments, popliteal lymph nodes. The footpad thickness was measured with a vernier caliper and the swelling caused by infection was determined by subtracting the thickness of the uninfected right hind footpad from that of the infected left hind footpad. To determine parasite burdens, infected footpads and/or popliteal lymph nodes were homogenized with steel mesh and 5-fold serially diluted with 15% FCS-containing Schneider's *Drosophila* medium and incubated at 26°C for 14 days. Emerged promastigotes were monitored and the parasite burden was calculated by the last dilution of promastigotes emerged (15).

Cell preparation

To obtain bone marrow (BM) cells, cells were prepared aseptically from femora. After removing RBCs by treatment with ammonium chloride solution, BM cells (2×10^7 cells ml^{-1}) were washed and suspended in PBS. To obtain splenocytes, spleen was first digested by injection of collagenase D solution (Boehringer Mannheim, Indianapolis, IN, USA) with a 26-G needle. After 10-min incubation at 37°C, spleen was minced with forceps. After pipetting, cell suspension was filtrated with nylon mesh to remove connective tissues. To obtain naive $CD4^+$ T cells from splenocytes, B and $CD8^+$ T cells were initially removed by using anti-CD8 and anti-B220 magnetic bead-conjugated mAbs. $CD4^+$ T cells were then positively collected with anti-CD4-conjugated magnetic beads and AutoMACS (Miltenyi Biotech) in accordance with the manufacturer's protocols. Purity of $CD4^+$ T cells was >95% in all experiments. Splenic DCs were obtained by magnetic separation of $CD11c^+$ cells from Rag-$2^{-/-}$ splenocytes. To obtain bone marrow-derived dendritic cells (BMDCs), BM cells from Rag-$2^{-/-}$ mice were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin and streptomycin, 10 mM HEPES and 200 U ml^{-1} of granulocyte macrophage colony-stimulating factor (PeproTech, London, UK). Ten days after culture, non-adherent cells were collected and $CD11c^+$ DCs were positively collected by magnetic sorting with AutoMACS. Purity of $CD11c^+$ cells was >90%.

In vitro *parasitization*

To prepare parasitized cells, Rag-$2^{-/-}$ splenocytes or BMDCs were mixed with *L. major* promastigotes (cells:promastigotes = 3:1) and incubated for 2 h at 37°C with rotation, washed three times to exclude extracellular promastigotes and suspended in PBS. To prepare DC-removed parasitized Rag-$2^{-/-}$ splenocytes, parasitized cells were mixed with anti-CD11c magnetic beads and $CD11c^+$ DCs were removed with VarioMACS (Miltenyi Biotech) in accordance with the manufacturer's protocols. Over 95% of $CD11c^+$ cells were removed from parasitized Rag-$2^{-/-}$ splenocytes. In some experiments, DC-removed parasitized Rag-$2^{-/-}$ splenocytes were mixed with parasitized $CD11c^+$ DCs at a ratio of 4:1 (determining by the percentage of $CD11c^+$ cells in Rag-$2^{-/-}$ splenocytes). To confirm the rate of parasitization, amastigotes in parasitized cells were directly observed on thin-blood smear specimens by Giemsa staining 3 days after parasitization. The infection rates of BALB/c and B10.D2 cells were nearly the same through all the experiments (data not shown).

T_h1/T_h2 cytokine assay

To determine the T_h1/T_h2 balance in mice, 4.0×10^5 splenocytes (2.0×10^6 ml^{-1}) were stimulated with freeze–thaw killed leishmanial antigens. Two days after stimulation, culture supernatants were harvested and stored at −80°C until performing ELISA. All ELISAs were performed with OptEIA™ ELISA sets (BD PharMingen) in accordance with the manufacturer's protocols.

Statistics

Student's *t*-test was applied to the results of footpad swelling and Mann–Whitney's *U*-test was applied to the results of parasite burdens.

Results

Genetic background of BM cells determines the outcome of L. major *infection*

To examine whether the outcome of *Leishmania* infection is determined by hematopoietic or non-hematopoietic cells, we

employed BM chimeras using Rag-$2^{-/-}$ mice on *L. major*-susceptible BALB/c and -resistant B10.D2 backgrounds. Rag-$2^{-/-}$ mice were lethally irradiated and transplanted with BM cells from BALB/c or B10.D2 WT mice. Two months after the transplantation of BM cells when the proportion of peripheral leukocytes was nearly the same as WT mice, BM chimeras were infected with *L. major*. As shown in Fig. 1, the outcome of infection such as footpad swelling, parasite burden and T_h1/T_h2 balance were reflected on the genetic background of BM donor. B10.D2-Rag-$2^{-/-}$ mice transplanted with BALB/c BM cells were unable to control *L. major* infection as revealed by progressive footpad swelling and high parasite burden in infected footpads as compared with the mice transplanted with B10.D2 BM cells (Fig. 1A and B). Moreover, these chimeras exhibited a T_h2 response as shown by the production of a small amount of IFN-γ and a large amount of IL-4 from splenocytes by re-stimulation with *L. major* antigens (Fig. 1C). In contrast, the BM chimeras based on BALB/c-Rag-$2^{-/-}$ mice transplanted with B10.D2 BM cells efficiently controlled the infection (Fig. 1D and E) and exhibited a characteristic T_h1 response (Fig. 1F) as compared with the mice transplanted with BALB/c BM cells. These results indicate that BM cells carry genetic factors controlling *L. major* infection.

Genetic background of $CD4^+$ T cells is minimally involved with the outcome

Next, to examine whether the outcome of *L. major* infection is determined by the genetic background of $CD4^+$ T cells (6), Rag-$2^{-/-}$ mice were reconstituted with $CD4^+$ T cells from naive B10.D2 or BALB/c WT mice and infected with *L. major*. B10.D2-Rag-$2^{-/-}$ mice reconstituted with $CD4^+$ T cells from either B10.D2 or BALB/c mice exhibited milder symptoms as compared with BALB/c-Rag-$2^{-/-}$ mice reconstituted with BALB/c $CD4^+$ T cells (Fig. 2). The ratios of IFN-γ and IL-4 produced by splenocytes were similar in B10.D2-Rag-$2^{-/-}$ mice reconstituted with B10.D2 and BALB/c $CD4^+$ T cells. On the other hand, such ratio was lower in BALB/c-Rag-$2^{-/-}$ mice reconstituted with BALB/c $CD4^+$ T cells. These results indicate that the outcome of disease is largely dependent upon the genetic background of recipient Rag-$2^{-/-}$ mice and, more specifically, non-T and -B cells among BM-derived cells.

Cells that first encounter parasites determine the outcome of infection

It has been suggested that the outcome of the disease upon infection with *L. major* is determined at an early phase of infection (7, 8). For example, the administration of cytokines or neutralizing antibodies against cytokines during the early phase of infection can drastically alter the disease outcome (7, 8). We therefore hypothesized that cells that initially encounter parasites determine the outcome of *L. major* infection. To examine the hypothesis, Rag-$2^{-/-}$ splenocytes containing macrophages and DCs were mixed with *L. major* promastigotes *in vitro* and parasitized cells were subcutaneously inoculated into the footpad of WT mice. Since macrophages are the major reservoir of *Leishmania* (2), leishmaniasis is induced by the inoculation of parasitized Rag-$2^{-/-}$ splenocytes.

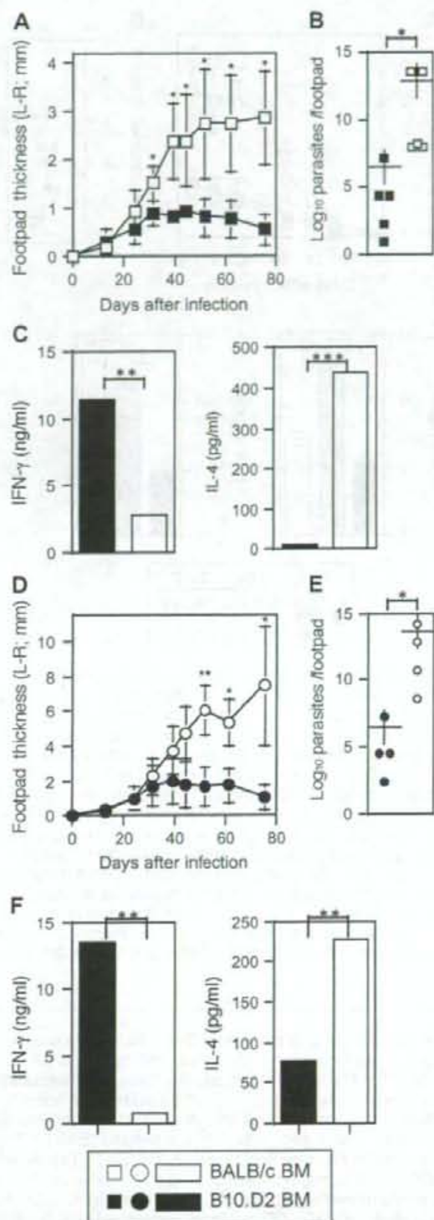


Fig. 1. The outcome of *Leishmania major* infection on BM chimeras is determined by the genetic background of BM cell donor. B10.D2-Rag-$2^{-/-}$ (A–C) and BALB/c-Rag-$2^{-/-}$ (D–F) mice were irradiated at 9 and 6 Gy, respectively, and reconstituted with 10^7 B10.D2-WT (closed symbols and bars) or BALB/c-WT BM (open symbols and bars) cells. Two months after transplantation, mice were administered with *L. major* promastigotes at left hind footpads. (A and D) Footpad swelling caused by *L. major* infection. (B and E) Parasite burdens in infected footpads. +, mean of each group. (C and F) IFN-γ and IL-4 production by splenocytes induced by parasite antigens. Statistical significance: $*P < 0.01$, $**P < 0.001$ and $***P < 0.0001$.

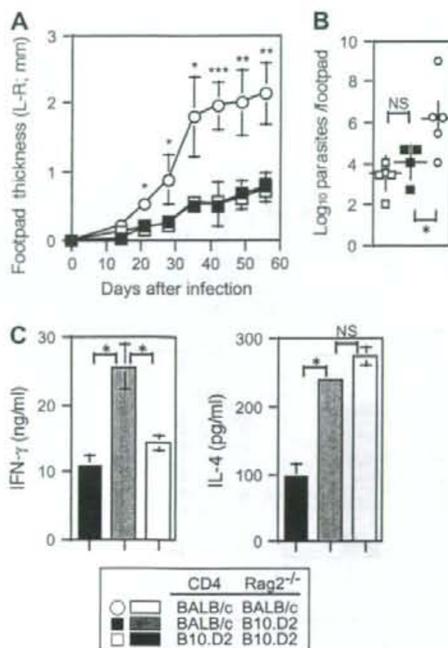
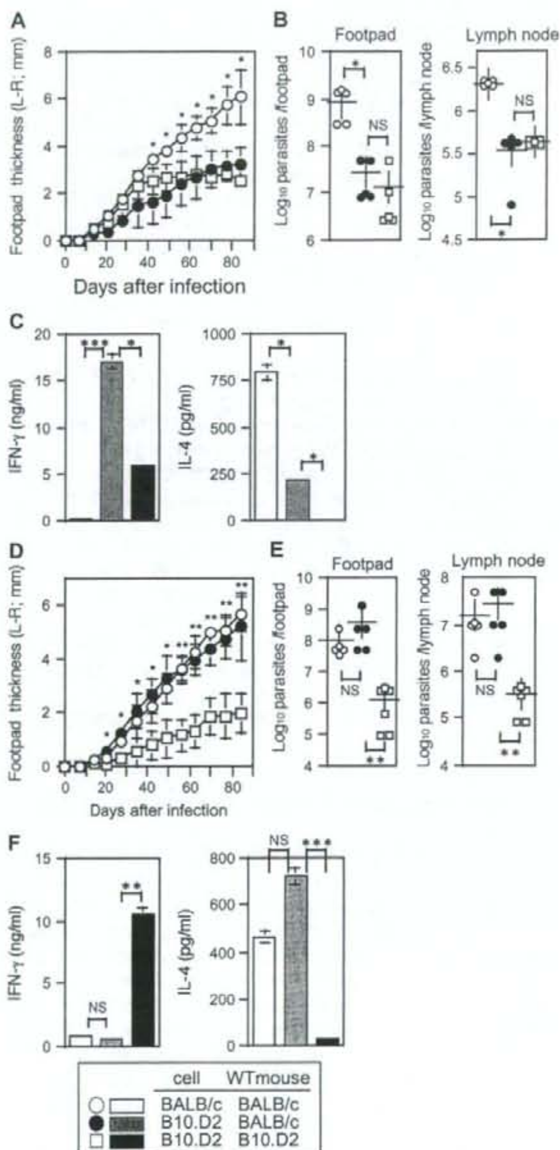


Fig. 2. The outcome of *Leishmania major* infection on Rag-$2^{-/-}$ mice reconstituted with $CD4^+$ T cells is determined by the genetic background of Rag-$2^{-/-}$ recipients. B10.D2-Rag-$2^{-/-}$ mice were reconstituted with 10^7 naive $CD4^+$ T cells collected from either B10.D2 (open squares and 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 $***P < 0.0001$.

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.

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



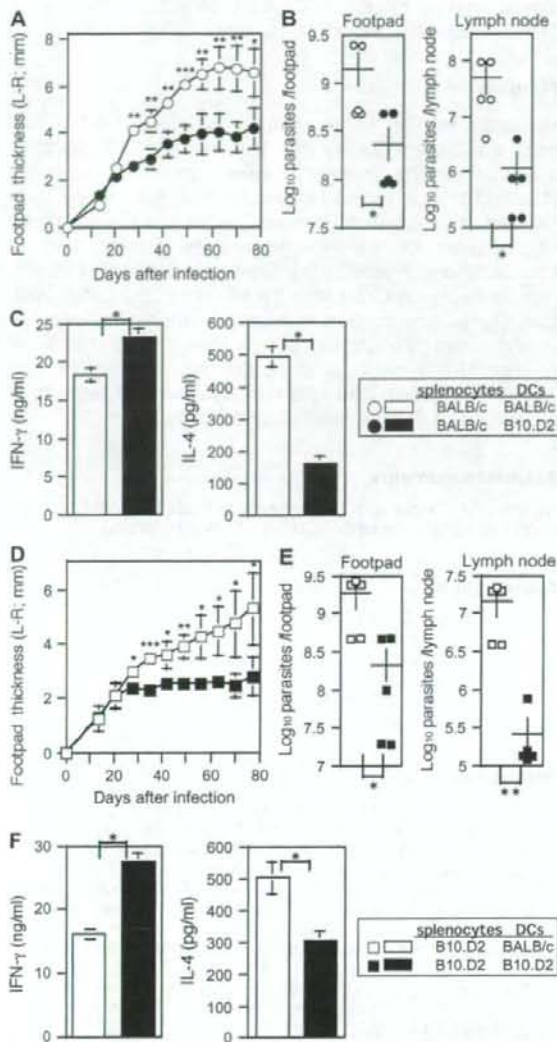


Fig. 4. *Leishmania major*-parasitized Rag-$2^{-/-}$ splenocytes mixed with parasitized B10.D2 CD11c$^+$ DCs lead BALB/c WT mice to mild outcome of the disease and T_h1 response. (A–C) DC-depleted *L. major*-parasitized BALB/c-Rag-$2^{-/-}$ splenocytes were mixed with parasitized CD11c$^+$ DCs from BALB/c (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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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.

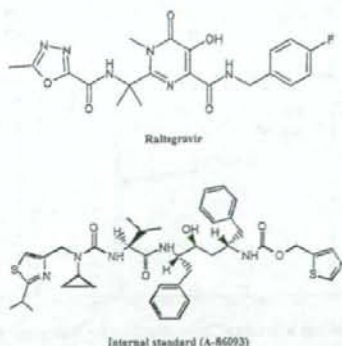

Fig. 1. Chemical Structures of Raltegravir and the Internal Standard A-86093

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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

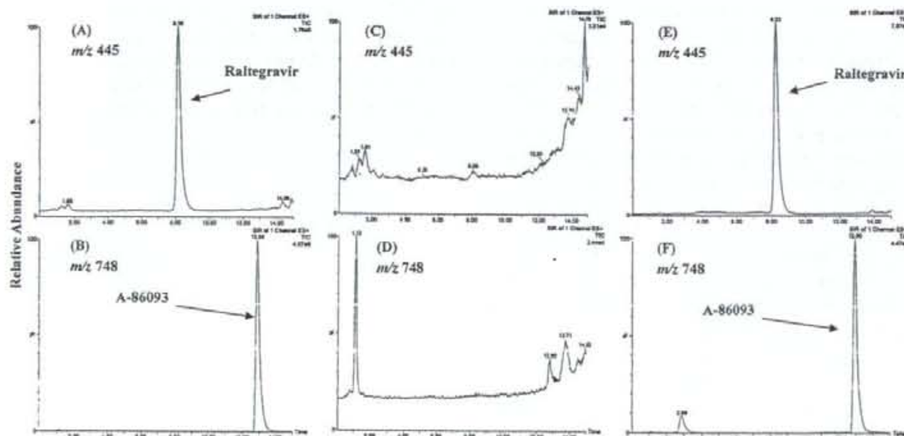

Fig. 2. Selected-ion Recording Chromatograms for Raltegravir and A-86093

(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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0047-1852/08/¥40/頁/JCLS

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[6]．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[8]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.