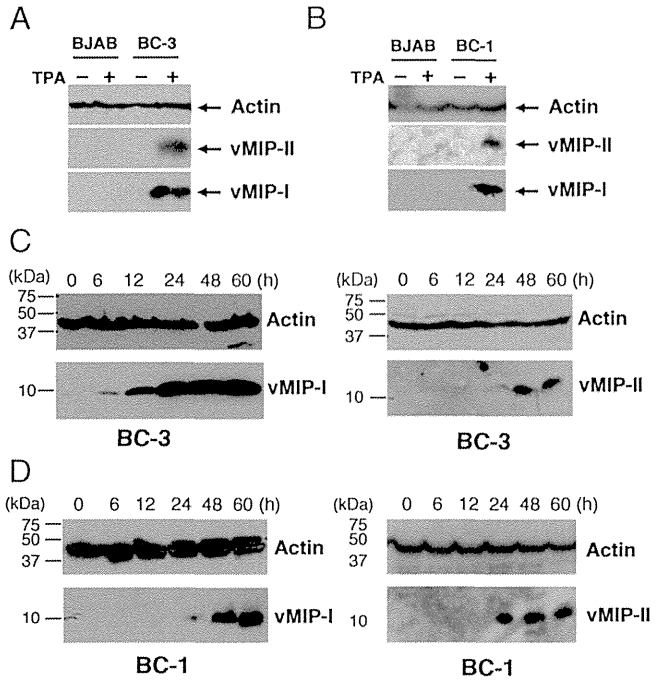

Fig. 3. Detection of vMIP-I and vMIP-II gene products in a KSHV-infected PEL cell line. BC-1 and BC-3 cells were treated with TPA for the indicated number of hours, and the whole-cell extract was prepared after the indicated time post-induction. vMIP-I and vMIP-II were detected by Western blotting and IFA with anti-vMIP-I and -vMIP-II antibodies. Western blot analysis of protein extracted from BC-3 and BJAB cells (A), and BC-1 and BJAB cells (B) with either the anti-vMIP-I or the anti-vMIP-II MAb. Arrows indicate actin, vMIP-I, and vMIP-II proteins. As expected, the estimated sizes of the vMIP-I and vMIP-II proteins, based on comparisons with the migration of molecular size markers, was around 10 kDa. Expression kinetics of vMIP-I (left panel) and vMIP-II (right panel) in TPA-treated BC-3 (C) and BC-1 (D) cells by Western blot analysis. BC-1 and BC-3 cells were harvested after 6, 12, 24, 48, and 60 h post-induction. The lysate was subjected to Western blot analysis as in (A).

GvM1-D3, GvM2-Full, GvM2-D1, GvM2-D2, and GvM2-D3 genes were generated by PCR using the following primer sets: vMIP-I-1F (5′-ATGAATTCCAGATGGCCCCCGTCCAC-3′) and vMIP-I-5R (5′-CCGTGTCGACCGTCTAAGCTATGGCAGGCAGC-3′); vMIP-I-2F (5′-ATGAATTCGCGGGGTCACTCGTGTCG-3′) and vMIP-I-5R; vMIP-I-3F (5′-ATGAATTCCCGCCCGTCCAAATTC-3′) and vMIP-I-5R; vMIP-I-4F (5′-ATGAATTCCCAAAACCCGGAGTTATTTTGC-3′) and vMIP-I-5R; vMIP-II-1F (5′-CGGAATTCGTTATGGACACCAAGGGC-3′) and vMIP-II-5R (5′-GGCAGTCGACTCTTCAGCGAGCAGTGACTG-3′); vMIP-II-2F (5′-GGGAATTCCTGGGAGCGTCCTGGCATAGAC-3′) and vMIP-II-5R; vMIP-II-3F (5′- AAGAATTCTTACCACAGGTGCTTCTGTCC-3′) and vMIP-II-5R; and vMIP-II-4F (5′-TGGAATTCAAGCCGGGTGTGATATTTTTG-3′) and vMIP-II-5R. The PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and confirmed by sequencing. The products were digested with the *Eco*RI and *Sal*I restriction enzymes and were cloned into pGEX-5X-1 (GE Healthcare). The PCR conditions for all products were as follows: 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min in a TP480 PCR thermal cycler (Takara Shuzo, Kyoto, Japan).

Immunization and generation of monoclonal Abs against vMIP-I and vMIP-II

In mice, anti-vMIP-I and -vMIP-II antibodies were raised against the GST-vMIP-I and GST-vMIP-II fusion protein, respectively. These GST fusion proteins were purified on a glutathione-Sepharose 4B column (GE Healthcare), and the GST-vMIP-I and the GST-vMIP-II fusion proteins were conjugated to keyhole limpet hemocyanin KLH (Calbiochem. Co., La Jolla, CA). Mice were initially immunized with 250 μg each of the purified GST-vMIP-I or -II fusion protein in Freund's complete adjuvant administered to the peritoneal cavity, and 200 μg of the antigen in Freund's incomplete adjuvant were injected again 14 and 28 days after the first injection. The mice were exsanguinated 7 days after the last injection. To generate MAbs against vMIP-I and vMIP-II, hybridomas were established by fusing splenocytes from the hyperimmune mice using a nonproducing myeloma cell line, Sp-2/0-Ag14 (ATCC, Manassas, VA). After selection in medium containing hypoxanthine-aminopterin-thymidine, cells secreting MAbs were screened by immunofluorescence assays (IFA). The TPA-induced and -uninduced BCBL-1 cells were fixed in acetone and exposed to supernatants of the hybrid cells. Clones secreting antibodies reactive with TPA-stimulated BCBL-1 cells were expanded and isolated by limiting dilutions.

Transfection analysis of vMIP-I and vMIP-II

To express the vMIP-I and vMIP-II proteins, 293/EBNA cells were transfected with pCAGGS-vMIP-I and -vMIP-II plasmids using TransIT-LT1 (Mirus Bio LLC, Madison, WI). The transfected cells were incubated for 48 h in DMEM supplemented with 10% FCS. The cells were harvested and lysed with lysis buffer (0.05 M Tris-HCl [pH 8.0], 0.15 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium-dodecyl sulfate [SDS]). The cell lysate was fractionated by electrophoresis on 16% polyacrylamide gel as described below.

Antibodies and Western blotting

The expression of vMIP-I and vMIP-II in BC-3 cells stimulated with TPA was determined with MAbs against vMIP-I and vMIP-II, respectively, as noted above. The concentration of proteins extracted from BC-3 cells was normalized using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). The samples were subjected to SDS-15% polyacrylamide gel electrophoresis under reducing conditions, and were electrophoretically transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1 h while being shaken at room temperature in PBS containing 0.05% Tween 20 and 5% w/v nonfat skim milk. The membranes were incubated with a primary antibody and were then incubated for 1 h with an appropriate dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). The primary antibody against actin, anti-actin (Ab-1) mouse MAb, was purchased from Merck (Merck KGaA, Darmstadt, Germany). The bound HRP-labeled antibodies were detected with a West Pico substrate kit for horseradish peroxidase (Thermo Fisher Scientific Inc).

IFA

BC-3 cells (10^7 cells) in RPMI 1640 medium with supplements were induced with 25 ng/ml TPA (Sigma Chemical Co., St. Louis, MO). The cells were collected after 0, 4, 8, 12, 24, 48, and 60 h for analysis of the expression kinetics, and for cellular localization analysis 48 h after exposure to TPA. The cells were washed in phosphate-buffered saline (PBS), pH 7.4, and spotted on glass slides. The spots were air-dried, then fixed in ice-cold acetone for 10 min. The cells were then washed with a washing buffer (PBS supplemented with 0.1% Triton X-100) for 15 min, and incubated with either an anti-vMIP-I or an anti-vMIP-II MAb (diluted 1:100 in IFA dilution buffer [PBS containing 2% bovine serum albumin, 0.2% Tween-20, and 0.05% NaN_3]) for 1 h at 37 °C. Then, the slides were washed with the washing buffer, and incubated for 1 h at room temperature with a pre-standardized diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Tago Immunologicals, Camarillo, CA). The slides were washed and stained with 4′, 6′-diamidino-2-phenylindole (DAPI) to detect nuclei and were mounted with 50% (v/v) glycerol in PBS. For formalin-fixed paraffin-embedded tissues, antigen retrievals were performed on the de-paraffined sections using citrate buffer. Alexa 488 or 568-conjugated

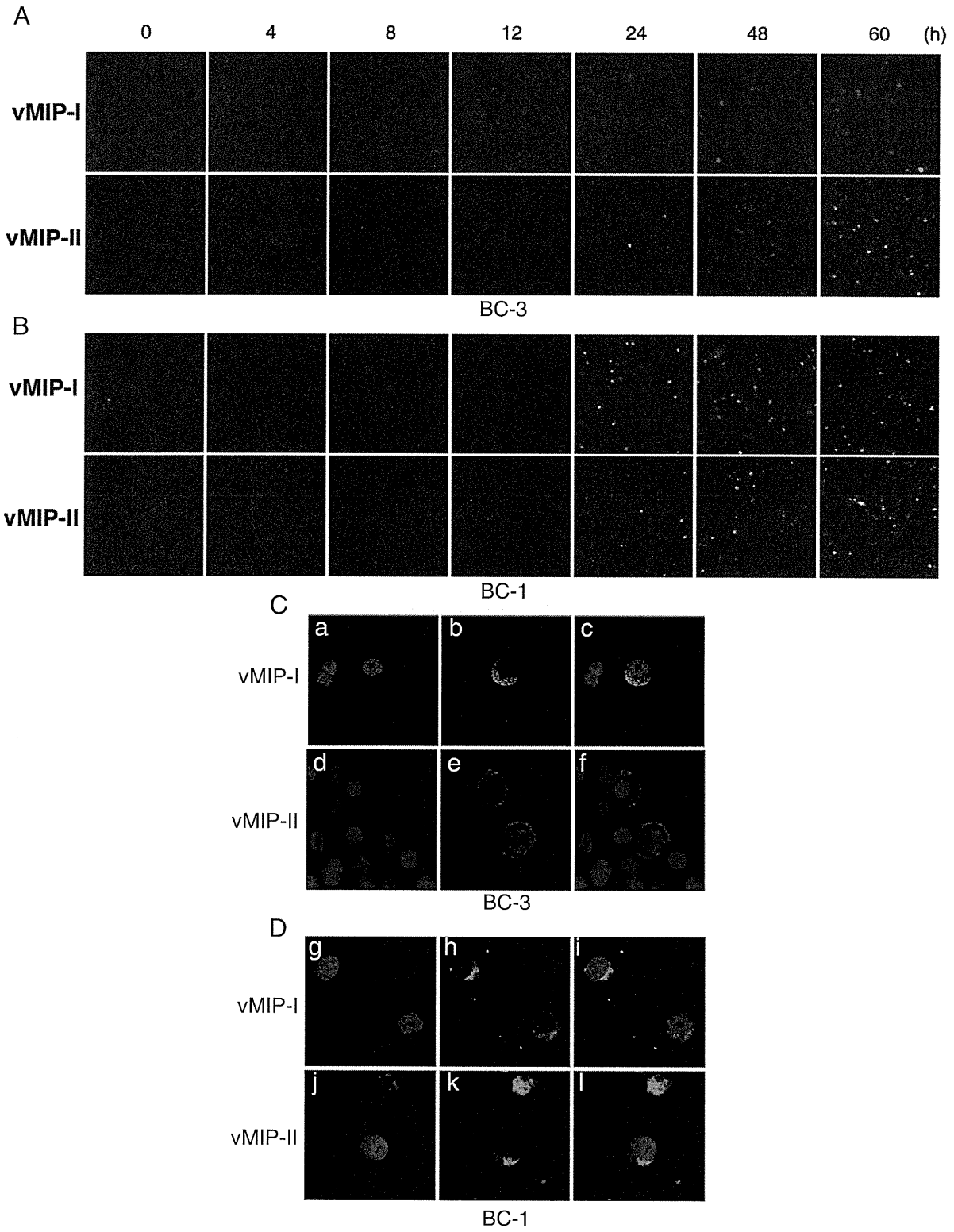

Fig. 4. Expression of vMIP-I and vMIP-II in BC-3 and BC-1 cells by IFA. After 4, 8, 12, 24, 48, and 60 h, BC-3 (A) and BC-1 (B) cells were labeled either with the anti-vMIP-I (upper) or the anti-vMIP-II (lower) MAb followed by goat anti-mouse FITC-conjugated Abs. FITC photomicrographs showing anti-vMIP-I and anti-vMIP-II immunoreactivity in BC-3 and BC-1 cells treated with TPA. (C) Cellular localization of vMIP-I and vMIP-II in BC-3 (C) and BC-1 (D) cells. The cells were stained with DAPI (a, d, g and j), and the localization of vMIP-I and vMIP-II was visualized by IFA with anti-vMIP-I or -vMIP-II MAbs (b, e, h and k); panel a and b, d and e, g and h, and j and k were merged (c, f, i and l). Fluorescence photomicrographs revealed anti-vMIP-I and -vMIP-II immunoreactivity using FITC-conjugated anti-mouse IgG MAb.

anti-mouse or rabbit antibodies (Invitrogen) were used as the secondary antibodies. Confocal microscopic analysis was performed (FV-1000, Olympus, Tokyo, Japan), and the contrast was adjusted before the images were exported as TIFF files to Adobe Photoshop.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissues from KS and MCD patients, and those from an animal model of KSHV-associated solid lymphoma

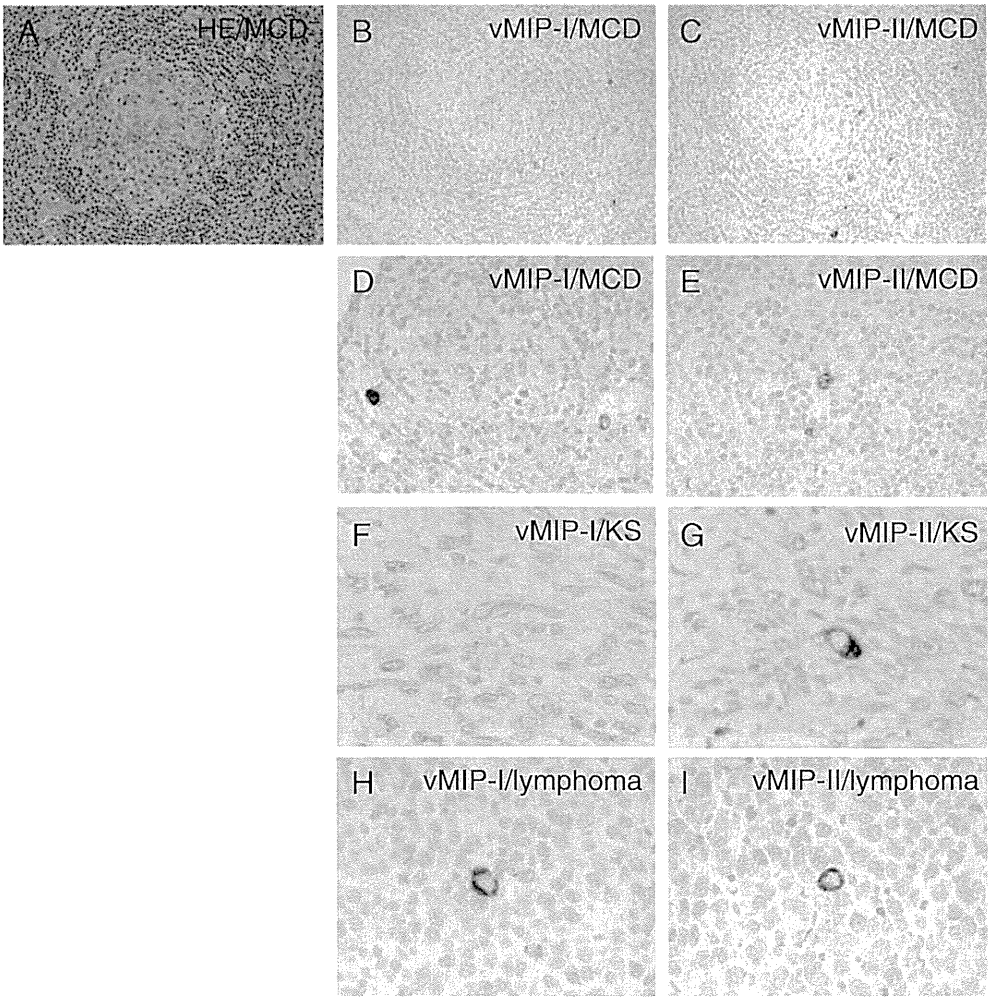

Fig. 5. Expression of vMIP proteins in KSHV-associated diseases. (A–C) Hematoxylin and eosin staining and immunohistochemistry for vMIPs in serial sections of a tissue sample from a patient with MCD. Brown stains indicate positive signals. The nucleus was counter-stained by hematoxylin. (D and E) Higher magnification view of vMIPs expression in an MCD case. Some large lymphocytes in the mantle zone were stained. (F and G) vMIP-I and vMIP-II expression in a KS sample. (H and I) Expression of vMIPs in an animal model of KSHV-associated lymphoma in SCID mice.

were sectioned and stained with hematoxylin and eosin (H&E). Immunohistochemistry of the serial sections was performed with either the anti-vMIP-I or -II MAb. For the second- and third- phase reagents used for immunostaining, a CSAII kit (DAKO, Copenhagen, Denmark) was used. An animal model of KSHV-associated solid lymphoma, which was established as described previously (Katano et al., 2000b), was also subjected to immunohistochemical analysis. Briefly, TY-1 cells were inoculated into the subcutaneous tissue of mice with severe combined immunodeficiency (SCID). One month after inoculation, lymphomas appeared in the subcutaneous region at the inoculation site. Lymphoma cells contained the KSHV genome, and expressed various viral proteins of KSHV (Katano et al., 2000b).

Table 1
Expression of vMIP-I and vMIP-II in MCD and KS tissue samples.

	KSHV proteins, (+)/total	
Cases	vMIP-I	vMIP-II
MCD	(3)/3	(3)/3
KS	(0)/5	(2)/8

Chemotaxis assays

Chemotaxis assays were performed as described previously (Nakano et al., 2003). Briefly, THP-1 cells were washed twice with chemotaxis buffer, 0.5% bovine serum albumin, 20 mM HEPES, pH 7.4, in RPMI 1640. Migration of cells was assessed in a cell culture chamber (Costar, Cambridge, MA), with the upper and lower compartments separated by a 3 μm pore size polycarbonate filter (???). The lower compartment of the chamber was filled with dilutions of vMIP-I, vMIP-II (R&D Systems, Minneapolis, MN) or with PBS alone, and/or with each 10 μg/ml anti-vMIP-I or -vMIP-II MAbs at a volume of 600 μl. The upper compartment contained 100 μl of THP-1 cell suspensions in chemotaxis buffer (10^5 cells/well). The chambers were then incubated for 4 hours at 37 °C, 5% CO_2, and spun at 300 x g, 4 °C, for 5 min. Finally, the cells from the lower compartment were counted.

Results

Specificity of the anti-vMIP-I MAb and the anti-vMIP-II MAb

In order to check specificity of the MAbs, we transfected vMIP-I and vMIP-II expression vectors (pCAGGS-vMIP-I, and -II) into 293/EBNA

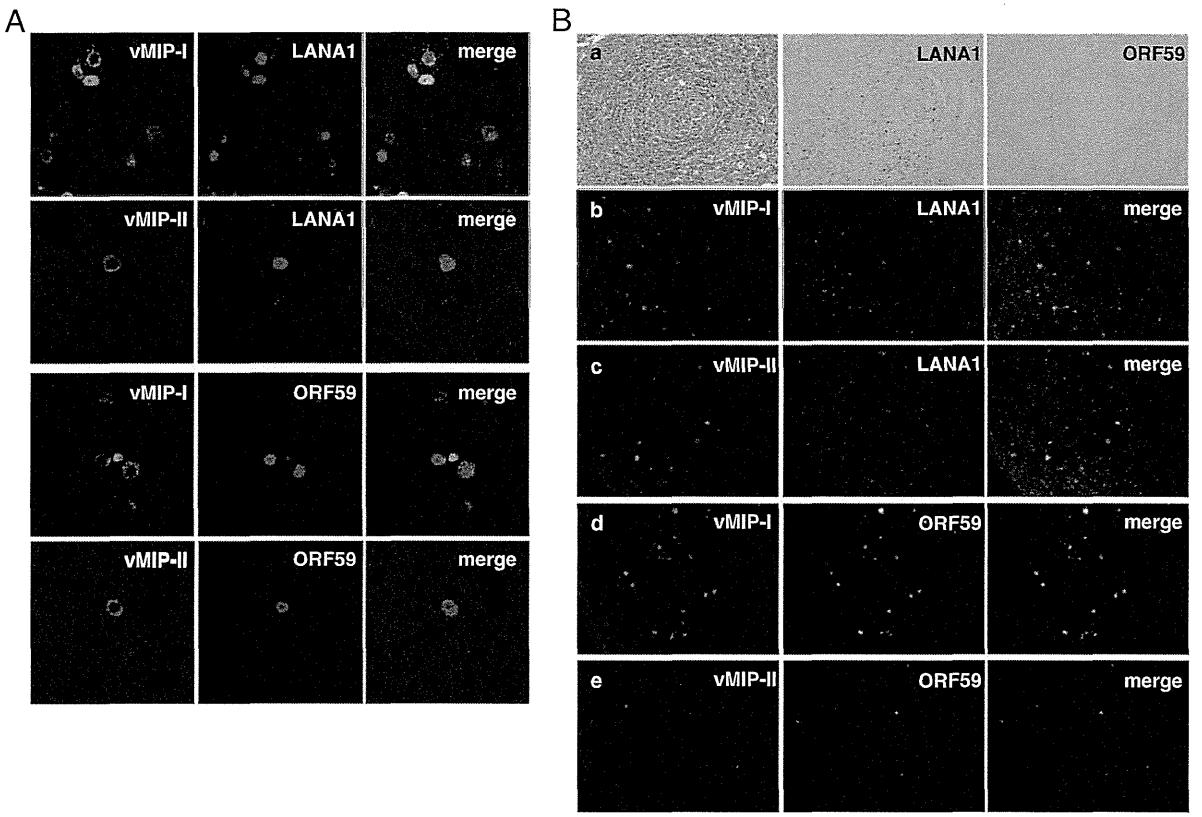

Fig. 6. (A) Expression of vMIPs. LANA1 and ORF59 in the animal model of KSHV-associated solid lymphoma by confocal microscopy. vMIPs were labeled with Alexa 488 (green). LANA1 (upper panels) and ORF59 (lower panels) were labeled with Alexa 568 (red). (B) Expression of vMIPs in MCD. (a) HE staining and immunohistochemistry of LANA1 and ORF59. (b–e) Immunofluorescence assay on MCD lesion. A germinal center is shown in the center of each panel. This case is KSHV-positive large B cell lymphoma arising in MCD (for interpretation of the color references in this article, the reader is referred to the online version).

cells, respectively. The total lysate of the transfected cells was subjected to Western blot analysis. vMIP-I and vMIP-II proteins were detected with anti-vMIP-I or vMIP-II MAbs, respectively (Fig. 1). These antibodies did not show cross-reactivity each other.

Epitope mapping of the anti-vMIP-I and anti-vMIP-II MAbs

We established hybridoma clones secreting MAbs against vMIP-I and vMIP-II, respectively. To map the regions of vMIP-I and vMIP-II where anti-vMIP-I and anti-vMIP-II antibody reacted, a series of GST-fused vMIP-I and vMIP-II deleted proteins were constructed as described in Fig. 2C and F, and used for Western blot analysis with an anti-GST antibody (Santa Cruz Biotechnology Inc), (Fig. 1A, D) and the anti-vMIP-I or the anti-vMIP-II (Fig. 1B, E) antibody, respectively. The results showed that all GST-vMIP-I and GST-vMIP-II fusion proteins interacted with the anti-GST antibody (Fig. 2A, D) and showed that GvM1-Full, GvM1-D1, and GvM1-D2 reacted with the anti-vMIP-I antibody, whereas GvM1-D3 did not (Fig. 1B), and GvM2-Full and GvM2-D1 reacted with the anti-vMIP-II antibody, whereas GvM2-D2, and GvM2-D3 did not (Fig. 2E). Thus, these results demonstrated that an anti-vMIP-I MAbs was successfully generated and suggest that the amino acid residues 61 to 95 of vMIP-I could be a major epitope reacted with the anti-vMIP-I antibody. On the other hand, the amino acid residues 24 to 42 of vMIP-II could be an epitope reacted with the anti-vMIP-II antibody.

Expression of vMIP-I and vMIP-II in the KSHV-infected PEL cell line

We tested vMIP-I and vMIP-II expression in KSHV and Epstein Barr virus (EBV) dually infected PEL cell lines (BC-1), KSHV infected PEL cell lines (BC-3) and in non-infected Burkitt's lymphoma cell line (BJAB), and detected them in TPA-stimulated BC-3 and BC-1 cells with developed antibodies, but not in BJAB cells non-stimulated BC-3 or BC-1 cells (Fig. 2A, B). In a KSHV infected PEL cells, BC-1 and BC-3, vMIP-I and vMIP-II were detected around at 10 kDa, which matches the size deduced from amino acids length (Fig. 3C, D). Actually, vMIP-I was detected from 6 hours post induction and vMIP-II was at 24 hours in BC-3 cells (Fig. 3C), and vMIP-I and vMIP-II were detected at 24 h in BC-1 cells (Fig. 3D). In the immunofluorescence microscopy, the number of vMIP-II expressing cells seemed to be more than that of vMIP-I in BC-3 cells (Fig. 4A, B). In order to analyze the cellular localization of vMIP-I and vMIP-II protein, BC-3 and BC-1 cells stimulated with TPA were doubly labeled with DAPI (Fig. 4C, a, d and D, g, j), and either the anti-vMIP-I MAb (Fig. 4C, b and D, h) or the anti-vMIP-II MAb (Fig. 4C, e and D, k). Merged images were shown in Fig. 4C, c, f, and D, i, l). The vMIP-I and the vMIP-II clearly showed cytoplasm and possibly membranes in TPA-induced BC-3 and BC-1 cells (Fig. 4C, b, e, and D, h, k).

Expression of vMIPs in KSHV-associated diseases

To know the expression of vMIPs in KSHV-associated diseases, immunohistochemistry for vMIPs was performed on pathological samples of eight KS cases, three MCD cases, and the animal model of KSHV-associated solid lymphoma (Fig. 5). Immunohistochemistry demonstrated that vMIP-I and vMIP-II were detected in some cells in the mantle zone of germinal center and the interfollicular zone in KSHV-positive MCD samples (Fig. 5A to E). Both vMIP-I and vMIP-II were detected predominantly in the cytoplasm of large lymphocytes. The numbers of positive cells varied among three MCD cases examined. On the other

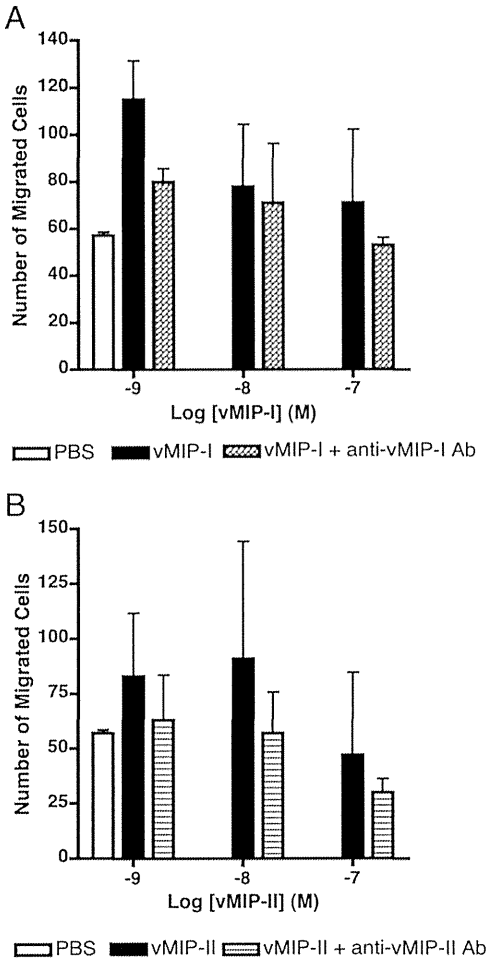

Fig. 7. Neutralizing activity of anti-vMIP-I and -vMIP-II MAbs. THP-1 cell migration in response to increased concentrations of vMIP-I and vMIP-II (1, 10, 100 nM), and the neutralizing activity of 10 μg/ml anti-vMIP-I and -vMIP-II MAbs against vMIP-I and vMIP-II were measured, as outlined in Materials and Methods, by using the transwell migration assay system. Various doses of vMIP-I and vMIP-II were tested for their ability to induce the chemotaxis of THP-1 cells. The data presented are from one experiment, and are representative of the triplicate experiments performed. The error bars indicate the standard deviations of three independent experiments.

hand, any positive signal of vMIP-I was not observed in all KS cases (Fig. 5F, G). vMIP-II was rarely detected in the cytoplasm of spindle cells in two KS cases at the nodular stage out of eight KS cases. In the samples of animal model of KSHV-associated solid lymphoma, both vMIP-I and vMIP-II were detected in the cytoplasm of a part of lymphoma cells (Fig. 5H, I). These data showed that vMIP-I and vMIP-II were expressed in cells in MCD and KSHV-associated lymphoma, but vMIP-II was rarely in KS (Table 1). To know the association of vMIPs expression with expression of other KSHV-encoded proteins, we examined immunofluorescence assay on KSHV-associated diseases. Since, all KSHV-infected cells express LANA1, vMIPs-positive cells were positive for LANA1. However, expression pattern of LANA1 showed diffuse nuclear staining in vMIPs-positive cells in the animal model of KSHV-associated solid lymphoma (Fig. 6A). Confocal microscopy revealed that vMIP-I stain showed usually cytoplasmic pattern, but rarely diffuse nuclear staining pattern *in vivo*. Almost all cells with vMIPs expression were also positive for ORF59 protein, a lytic protein of KSHV. IFA also demonstrated that vMIPs-positive cells expressed LANA1 at various levels in MCD clinical samples (Fig. 6B, a to c). A large portion of vMIPs-positive cells also expressed ORF59 protein in MCD (Fig. 6B, d, e). These data suggest that vMIPs are expressed by cells with KSHV-lytic infection in KSHV-associated MCD and lymphoma.

Neutralization of vMIP-I and vMIP-II by anti-vMIP-I and anti-vMIP-II MAbs

We examined whether the anti-vMIP-I and anti-vMIP-II MAbs could neutralize the chemoattractant of vMIP-I and vMIP-II to induce the migration of THP-1 cells. As expected, vMIP-I and vMIP-II induced migration of THP-1 cells (Fig. 7A, B), but not with PBS alone. However, anti-vMIP-I and anti-vMIP-II MAbs inhibited respective vMIP-I and vMIP-II-induced cell migration of THP-1 cells at 10 μg/ml final concentration.

Discussions

It was known that KSHV encodes three chemokine genes of the so-called viral macrophage inflammatory proteins: *vMIP-I*, *vMIP-II*, and *vMIP-III* in the genome. Analysis of the translated amino acid sequence indicate that the vMIP-I and vMIP-II gene have four conserved cysteines capable of forming two essential disulfide bonds (first cysteine and third cysteine, and second cysteine and fourth cysteine). The family of chemokines comprises CC, CXC, C, and CX_3C subfamilies. The vMIP-I and vMIP-II have four cysteines, the first two of which are found in the sequence of CC, which correspond to the CC profile. These gene products were expressed in the phase of KSHV lytic infection (Moore et al., 1996; Sun et al., 1999). Both vMIP-I and vMIP-II were expressed in a KSHV-infected cell lines, BC-3, which had been treated with TPA. Mono-specific polyclonal Abs against vMIP-I and vMIP-II have been described in previous studies that investigated the localization of vMIPs in PEL cells (Nakano et al., 2003). In the present study, we developed the respective MAbs that reacted either with KSHV vMIP-I or vMIP-II. We first applied these MAbs against KSHV vMIP-I and vMIP-II to detect KSHV-infected BC-3 and BC-1 cells by Western blotting and immunofluorescence assay. The Western blot analysis revealed that both the anti-vMIP-I and the anti-vMIP-II MAbs reacted to the 10-kDa proteins considered specific to the respective vMIP protein. The anti-vMIP-I MAb was shown to be reactive with the epitopes in the middle of the protein (sequence, PPVQILKEWYPTSPAC), and the epitope of the anti-vMIP-II MAb was shown to be reactive at the N-terminal end (sequence, LGASWHRPDKCCLGYQKRP). Further immunofluorescence analysis of the cellular localization of both vMIP-I and vMIP-II with anti-vMIP-I and anti-vMIP-II MAb showed a cytoplasmic pattern of expression in BC-3 and BC-1 cells. As the results indicated that these gene products were expressed in the cytoplasm, it might be located at the KSHV-infected BC-3 or BC-1 cells membrane prior to secretion. An investigation of the antigenic specificities of MAbs against KSHV vMIP-I and vMIP-II in MCD and KS patients has not yet been reported. Here, immunohistochemical analysis detected only vMIP-II in samples from both KS and MCD patients, but vMIP-I was not detected in KS cases: however, both vMIP-I and vMIP-II proteins were expressed in some cells in the interfollicular zone of MCD tissues. Lytic proteins of the KSHV such as K8, RTA, and ORF59 have been detected in large lymphocytes in the mantle zone of MCD cases (Dupin et al., 1999; Katano et al., 2000a). The expression of vMIPs showed a similar pattern to that of the lytic proteins in MCD tissues. In contrast, lytic protein expression, including that of vMIPs, was rare in the KS lesions (Abe et al., 2006). In the present study, we demonstrated that vMIPs were expressed in the cells expressing ORF59 protein. Thus, our data clearly indicated that the expression of vMIPs is associated with lytic infection in individual cells affected by KSHV-associated diseases. Human monocytic cell line THP-1 respond to various chemokines suggesting that they express receptors for these chemokines (Wang et al., 1993). Previous study, vMIP-I and vMIP-II were shown chemotaxis in THP-1 cells (Nakano et al., 2003). It has been reported that vMIP-I acts as a specific agonist for CC chemokine receptor 8 (CCR8) (Dairaghi et al., 1999; Endres et al., 1999) and vMIP-II shows a Ca^{2+} flux as a specific agonist for CCR3 (Boshoff et al., 1997). Our data showed anti-vMIP-I and anti-vMIP-II MAbs were able to neutralize vMIP-I- and vMIP-II-mediated chemotaxis in THP-1 cells. However, neutralizing activities

of anti-vMIP-I MAb was apparently low, even the addition of 10 μg/ml MAbs. These findings support the assumption that anti-vMIP-I and –vMIP-II MAbs-blocked chemotaxis in THP-1 cells act through binding to the certain amino acid residue of vMIP-I and vMIP-II.

In summary, MAbs developed specifically for this series were used to detect vMIP-I and vMIP-II in MCD and KS tissues, which may account for certain clinical features of MCD and KS. To gain a better understanding of these important viral genes, additional studies will be needed that focus on revealing vMIP-I and vMIP-II expression profiles during lytic infection. Taken together, these studies provide an insight into the pathogenesis of the contribution of vMIP-I and vMIP-II to the lytic induction of KSHV. These MAbs could serve as useful tools to clarify the pathogenesis of KSHV-related diseases.

Acknowledgments

This study was supported by a grant for Research on Publicly Essential Drugs and Medical Devices from the Japan Health Sciences Foundation (SAA4832), Health and Labor Sciences Research Grants (to HK, No. H23-AIDS-Ippan-002) from the Ministry of Health and by a grant from PRESTO of the Japan Science and Technology Corporation (200154023).

References

Abe, Y., Matsubara, D., Gatanaga, H., Oka, S., Kimura, S., Sasao, Y., Saitoh, K., Fujii, T., Sato, Y., Sata, T., Katano, H., 2006. Distinct expression of Kaposi's sarcoma-associated herpesvirus-encoded proteins in Kaposi's sarcoma and multicentric Castleman's disease. Pathol. Int. 56, 617–624.

Arvanitakis, L., Mesri, E.A., Nador, R.G., Said, J.W., Asch, A.S., Knowles, D.M., Cesarman, E., 1996. Establishment and characterization of a primary effusion (body cavity-based) lymphoma cell line (BC-3) harboring kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in the absence of Epstein-Barr virus. Blood 88, 2648–2654.

Benelli, R., Barbero, A., Buffa, A., Aluigi, M.G., Masiello, L., Morbidelli, L., Ziche, M., Albini, A., Noonan, D., 2000. Distinct chemotactic and angiogenic activities of peptides derived from Kaposi's sarcoma virus encoded chemokines. Int. J. Oncol. 17, 75–81.

Boshoff, C., Endo, Y., Collins, P.D., Takeuchi, Y., Reeves, J.D., Schweickart, V.L., Siani, M.A., Sasaki, T., Williams, T.J., Gray, P.W., Moore, P.S., Chang, Y., Weiss, R.A., 1997. Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. Science 278, 290–294.

Cesarman, E., Chang, Y., Moore, P.S., Said, J.W., Knowles, D.M., 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N. Engl. J. Med. 332, 1186–1191.

Chang, Y., Cesarman, E., Pessin, M.S., Lee, F., Culpepper, J., Knowles, D.M., Moore, P.S., 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 266, 1865–1869.

Chen, S., Bacon, K.B., Li, L., Garcia, G.E., Xia, Y., Lo, D., Thompson, D.A., Siani, M.A., Yamamoto, T., Harrison, J.K., Feng, L., 1998. In vivo inhibition of CC and CX3C chemokine-induced leukocyte infiltration and attenuation of glomerulonephritis in Wistar–Kyoto (WKY) rats by vMIP-II. J. Exp. Med. 188, 193–198.

Dairaghi, D.J., Fan, R.A., McMaster, B.E., Hanley, M.R., Schall, T.J., 1999. HHV8-encoded vMIP-I selectively engages chemokine receptor CCR8. Agonist and antagonist profiles of viral chemokines. Biol. Chem. 274, 21569–21574.

Dupin, N., Fisher, C., Kellam, P., Ariad, S., Tulliez, M., Franck, N., van Marck, E., Salmon, D., Gorin, I., Escande, J.P., Weiss, R.A., Alitalo, K., Boshoff, C., 1999. Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Proc. Natl. Acad. Sci. U. S. A. 96, 4546–4551.

Endres, M.J., Garlisi, C.G., Xiao, H., Shan, L., Hedrick, J.A., 1999. The Kaposi's sarcoma-related herpesvirus (KSHV)-encoded chemokine vMIP-I is a specific agonist for the CC chemokine receptor (CCR)8. J. Exp. Med. 189, 1993–1998.

Katano, H., Sato, Y., Kurata, T., Mori, S., Sata, T., 2000a. Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. Virology 269, 335–344.

Katano, H., Suda, T., Morishita, Y., Yamamoto, K., Hoshino, Y., Nakamura, K., Tachikawa, N., Sata, T., Hamaguchi, H., Iwamoto, A., Mori, S., 2000b. Human herpesvirus 8-associated solid lymphomas that occur in AIDS patients take anaplastic large cell morphology. Mod. Pathol. 13, 77–85.

Kledal, T.N., Rosenkilde, M.M., Coulin, F., Simmons, G., Johnsen, A.H., Alouani, S., Power, C.A., Luttichau, H.R., Gerstoft, J., Clapham, P.R., Clark-Lewis, I., Wells, T.N., Schwartz, T.W., 1997. A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. Science 277, 1656–1659.

Miller, G., Heston, L., Grogan, E., Gradoville, L., Rigsby, M., Sun, R., Shedd, D., Kushnaryov, V.M., Grossberg, S., Chang, Y., 1997. Selective switch between latency and lytic replication of Kaposi's sarcoma herpesvirus and Epstein-Barr virus in dually infected body cavity lymphoma cells. J. Virol. 71, 314–324.

Moore, P.S., Boshoff, C., Weiss, R.A., Chang, Y., 1996. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. Science 274, 1739–1744.

Nakano, K., Isegawa, Y., Zou, P., Tadagaki, K., Inagi, R., Yamanishi, K., 2003. Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded vMIP-I and vMIP-II induce signal transduction and chemotaxis in monocytic cells. Arch. Virol. 148, 871–890.

Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108, 193–199.

Schalling, M., Ekman, M., Kaaya, E.E., Linde, A., Biberfeld, P., 1995. A role for a new herpes virus (KSHV) in different forms of Kaposi's sarcoma. Nat. Med. 1, 707–708.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M.F., Clauvel, J.P., Raphael, M., Degos, L., et al., 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood 86, 1276–1280.

Sun, R., Lin, S.F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., Miller, G., 1999. Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. J. Virol. 73, 2232–2242.

Wang, J.M., McVicar, D.W., Oppenheim, J.J., Kelvin, D.J., 1993. Identification of RANTES receptor on human monocytic cells: competition of binding and desensitization by homologous chemotactic cytokines. J. Exp. Med. 177, 699–705.

症例報告

エファビレンツ，テノホビル/エムトリシタビンを大量服用した症例の血中濃度推移について

大石　裕樹[1,4]，安藤　仁[2]，高橋　昌明[3]，髙濱宗一郎[4]，
喜安　純一[4]，南　留美[4]，石橋　誠[1]，山本　政弘[4]

[1] 国立病院機構九州医療センター薬剤科，[2] 聖マリアンナ医科大学難病治療研究センター，
[3] 国立病院機構名古屋医療センター薬剤科，[4] 国立病院機構九州医療センター臨床研究センター

目的：エファビレンツ（EFV），テノホビル/エムトリシタビン（TDF/FTC）の大量服用後に血中濃度を経時的に測定した症例を経験したので報告する。

症例：29歳，男性。EFV（200 mg）を90錠，TDF/FTCを30錠服用し，緊急入院となった。入院後，薬用炭の投与とハイドレーションを行いながら，EFV，TDFについて経時的に血中濃度を測定した。EFVの血中濃度は，600 mgを単回投与したときのトラフ値と比較して24時間値で約8倍まで上昇し，96時間値で同等の値まで低下した。TDFの血中濃度は，300 mgを単回投与したときのトラフ値と比較して，48時間値で約12倍まで上昇し，同等の値まで減少するのに96時間を要した。検査値異常としてCK-MB値の上昇が観察された。

考察：EFVは著しい消失時間の延長は観察されず，TDFは吸収過程の遷延が観察された。EFVは薬用炭に吸着されやすく，親水性の高いTDFにはハイドレーションが有効であったと考えられる。CK-MB値の上昇は，心筋障害による可能性が高いと考えられた。

キーワード：ART，大量服用，EFV，TDF

日本エイズ学会誌 **14**：42-45，2012

緒　　言

エファビレンツ（EFV）は，非核酸系逆転写酵素阻害薬に分類される抗HIV薬である。EFVは優れた抗ウイルス効果を示し，1日1回の投与が可能で，吸収に食事の影響をうけない。またテノホビル/エムトリシタビン（TDF/FTC）は2種類の核酸系逆転写酵素阻害剤の合剤で，こちらも1日1回投与が可能である。これらの薬剤は抗HIV作用に優れることと服薬の簡便さから，さまざまなガイドラインにおいて初回治療に推奨される組合せとして設定され，Antiretroviral therapy（ART）のなかでも使用頻度の高い薬剤である。

両剤とも重篤な副作用発現は稀であるが，EFVでは抑うつ状態，うつ症状の悪化，めまい，健忘，錯乱など，精神神経系の副作用が発現しやすいことが知られている[1]。またTDF/FTCは，腎機能低下症例や大量服用でTDFによる腎障害が発現しやすくなることが知られている[2]。

今回われわれは，EFV＋TDF/FTCによるARTを継続中に大量服用した症例を経験し，大量服用後の血中薬物濃度を経時的に測定した。EFVとTDF/FTCを大量服用後に血中濃度を経時的に測定した報告は少なく，臨床上有用なデータであると考えたので報告する。

著者連絡先：大石裕樹（〒810-8563　福岡市中央区地行浜1-8-1　独立行政法人国立病院機構九州医療センター薬剤科）

2011年6月28日受付；2011年9月30日受理

症　　例

症例：29歳，男性。体重51 kg。2009年4月，HIV感染症と診断。診断時，HIV-RNA 12,000 copies/mL，CD4+リンパ球数（CD4数）は209 cells/mm^{3}であった。CD4数の低下が顕著であったため，同年6月よりEFV＋TDF/FTCによるARTが開始となった。その他の併用薬はなかった。アドヒアランスは良好であり，HIV-RNA検出感度以下，CD4数600～700 cells/mm^{3}で推移した。2010年1月，自殺企図にて自宅でEFV（200 mg）を90錠，TDF/FTCを30錠服用後，下痢，嘔吐出現し，知人に連れられ当院を受診した。自殺企図・抗HIV薬の大量服用であることから，緊急入院となった。

薬剤の排泄促進と腎保護の目的で，大量服用から192時間までは生理食塩水によるハイドレーションを行った。白色便が続いていたため，薬剤が消化管内に残存している可能性を考え，大量服用から22時間後に薬用炭（30 g）を投与した。自殺企図の原因がEFVによる精神神経系の副作用発現であった可能性も否定できず，TDFの血中濃度が上昇することによる腎機能障害も危惧されたため，EFV，TDF/FTCによるARTの継続は困難であると判断し，代替薬として大量服用から24時間後にラルテグラビル，72時間後にアバカビル/ラミブジンを開始した。

The Journal of AIDS Research Vol. 14 No. 1 2012

EFV の血中濃度を大量服用後，24，48，72，96，120，144，168 時間で測定したところ，それぞれの測定値は，6,860，4,090，1,960，860，510，320，180 ng/mL であった（図 1）。TDF の血中濃度は大量服用から 24，48，72，96 時間で測定し，それぞれの測定値は，310，370，40，30 ng/mL であった（図 2）。

EFV の血中濃度に影響する因子として代謝酵素 CYP2B6 の遺伝子多型（G516T）が知られており，変異型ホモ（T/T）の保有者では EFV の代謝が減弱し，野生型（G/G）の患者と比較して最高血中濃度が 2〜4 倍に上昇し，消失半減期が約 2 倍となる[3]。患者の同意を得て *CYP2B6* (*G516T*) 遺伝子多型の解析を行ったところ，本症例は，野生型（G/G）であり，EFV の薬物動態に遺伝子多型の影響はなかったと考えられる。遺伝子多型の解析については九州医療センター倫理審査委員会での承認を得ている（受付番号：09-56）。

また，経過観察中，TDF による腎機能の低下が危惧されたが，大量服用後 120 時間まで Scr は 0.5〜0.7 mg/dL で推移した。また，大量服用後 120 時間での Ccr は 72.9 mL/min であった。本症例では腎機能の低下はなかったと考えられる。

検査値異常として一過性に CK-MB 値の上昇が観察され，CK-MB の測定値は大量服用後 19，43，67，139，187 時間で 71，110，76，32，20 U/L（基準値 10〜21 U/L）であった。また，同時点での CK 値は，それぞれ 68，83，72，59，48 IU/L（基準値 62〜287 IU/L）で，大きな変動はなかった。その他の検査値にも大きな変動は見られなかった。その後，有害事象は発現せず，2010 年 2 月に退院となった。

考　　察

本症例で測定された EFV の血中濃度は，600 mg を単回投与したときのトラフ値（900 ng/mL）と比較して 24 時間値で約 8 倍（6,860 ng/mL）まで上昇したが，96 時間値では 860 ng/mL まで低下した。また EFV（600 mg）服薬後の血中濃度の推移（未発表データ）と比較しても 120 時間以降の血中濃度はほぼ同等であったことから，本症例では大量服用による消失時間の著しい延長はなかったと考える。

TDF の血中濃度は，300 mg を単回投与したときのトラフ

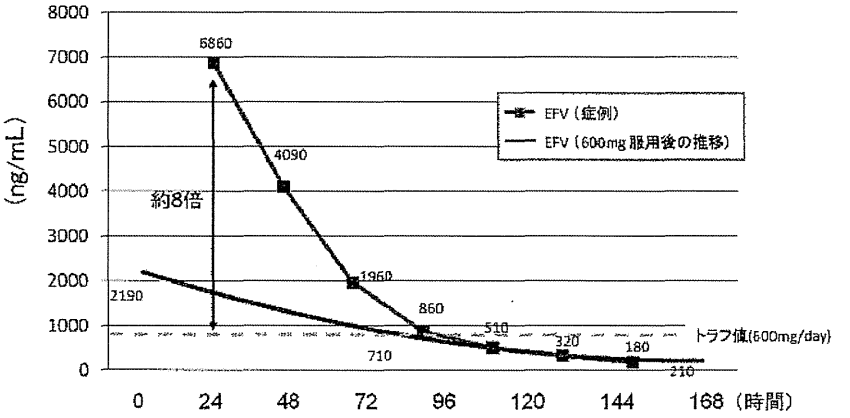

図 1　血中濃度の推移（EFV）

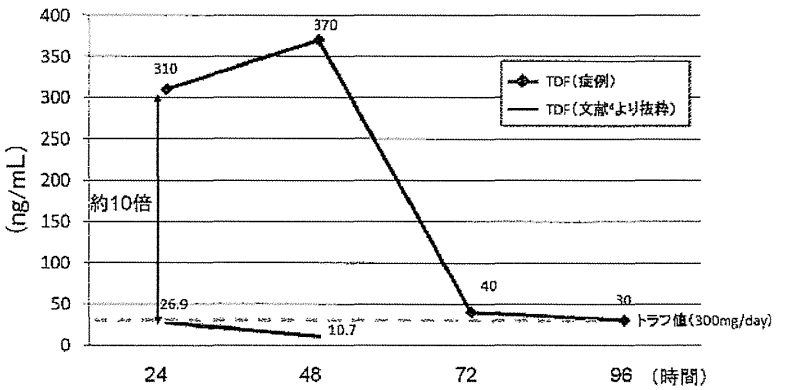

図 2　血中濃度の推移（TDF）

値（30 ng/mL）と比較して，24 時間値で約 10 倍（310 ng/mL），48 時間値では約 12 倍（370 ng/mL）まで上昇し，30 ng/mL まで減少するのに 96 時間を要した。中道[4]によって報告された TDF 300 mg 服用後の血中濃度の推移と比較しても，TDF の血中薬物動態には消失時間の延長がみられた。

EFV は脂溶性の高い薬剤で，血中でのタンパク結合率は 99.5% と高く，大部分が糞中へ排泄される[5]。そのため，透析では除去されないと考えられている。TDF は親水性が高く，大部分が尿中へ排泄され，透析によって除去される[6]。本症例では透析は行っていないが，薬剤の排泄促進と腎保護を目的としてハイドレーション，薬用炭の投与を行った。現在までにハイドレーションや薬用炭の投与が EFV，TDF の排泄を促進したという報告はないが，脂溶性の高い EFV は薬用炭によって吸着され，親水性の高い TDF の排泄はハイドレーションにより促進されると推察される。前述のとおり，本症例は大量服用から 22 時間まで白色便を呈しており，薬用炭を投与した時点では大量服用された薬剤の多くが消化管内に残存していたと考えられる。消化管内に残存した EFV が薬用炭に吸着され，吸収が抑制されたことで，EFV 消失時間の著しい延長を回避することができたと考える。また，TDF の T_{max} が約 1 時間であるにもかかわらず[6]，本症例の TDF 血中濃度は大量服用後 48 時間でピークとなった。この現象の一因として，親水性である TDF が薬用炭によって十分には除去されず，消化管に残存した TDF の吸収が遷延した可能性が考えられる。その後，TDF 血中濃度は速やかに低下しており，TDF の排泄促進という点で，ハイドレーションの有効性が示唆された。

経過観察中に一過性の上昇が観察された CK-MB 値は，主に心筋の障害で上昇する。しかしながら，現在までに EFV，TDF/FTC が副作用として心筋障害を招くという報告はなく，EFV や TDF/FTC の服用時に CK-MB 値の変動を観察した報告もない。CK-MB 値は，まれに骨格筋の障害により上昇するが，同時点での CK 値が正常範囲であったことから，本症例で観察された CK-MB 値の上昇が骨格筋由来であったとは考えにくい。したがって，大量服用後の CK-MB 値の上昇は，心筋障害によるものであった可能性が高く，EFV や TDF/FTC の大量服用が心筋障害を引き起こす可能性を示唆する。よって，EFV や TDF/FTC の大量服用時には心筋障害のモニタリングが必要ではないかと考える。

結　　語

EFV，TDF/FTC を大量服用した症例を経験し，大量服用後の血中濃度を経時的に測定した。EFV の消失時間に大きな変化は見られず，TDF の消失時間は延長した。本症例においては，薬用炭による EFV の吸収抑制と TDF の排泄促進を目的としたハイドレーションの有効性が示唆された。また EFV，TDF/FTC の大量服用に関連して心筋障害を疑う CK-MB 値の上昇が観察されたが，重篤な有害事象は観察されなかった。

文　　献

1）Haas DW, Ribaudo HJ, Kim RB, Tierney C, Wilkinson GR, Gulick RM, Clifford DB, Hulgan T, Marzolini C, Acosta EP : Pharmacogenetics of efavirenz and central nervous system side effects : An Adult AIDS Clinical Trials Group Study. AIDS 18 : 2391–2400, 2004.

2）Nelson MR, Katlama C, Montaner JS, Cooper DA, Gazzard B, Clotet B, Lazzarin A, Schewe K, Lange J, Wyatt C, Curtis S, Chen SS, Smith S, Bischofberger N, Rooney JF : The safety of tenofovir disoproxil fumarate for the treatment of HIV infection in adults : The first 4 years. AIDS 21 : 1273–1281, 2007.

3）Ribaudo HJ, Haas DW, Tierney C, Kim RB, Wilkinson GR, Gulick RM, Clifford DB, Marzolini C, Fletcher CV, Tashima KT, Kuritzkes DR, Acosta EP : Pharmacogenetics of plasma efavirenz exposure after treatment discontinuation : An Adult AIDS Clinical Trials Group Study. Clin Infect Dis 42 : 401–407, 2006.

4）中道昇：ビリアード®錠 300 mg の日本人健康成人男性を対象とした薬物動態試験．新薬と臨牀 54：941–948，2005.

5）ストックリン®錠 200 mg 医薬品インタビューフォーム（2011 年 3 月）.

6）ビリアード®錠 300 mg 医薬品インタビューフォーム（20011 年 1 月）.

The Journal of AIDS Research Vol. 14 No. 1 2012

Plasma Concentrations of Efavirenz and Tenofovir in Overdose

Yuki OHISHI[1,4], Hitoshi ANDO[2], Masaaki TAKAHASHI[3], Soichiro TAKAHAMA[4], Jyunichi KIYASU[4], Rumi MINAMI[4], Makoto ISHIBASHI[1], and Masahiro YAMAMOTO[4]

[1] Department of Pharmacy, National Hospital Organization Kyusyu Medical Center,
[2] Department of Rare Disease Research, Institute of Medical Science,
St Marianna University School of Medicine,
[3] Department of Pharmacy, National Hospital Organization Nagoya Medical Center,
[4] Department of Clinical Research Center, National Hospital Organization Kyusyu Medical Center

Case : A 29-year-old Japanese male with HIV infection had been followed up at Kyusyu Medical Center under ART with Efavirenz (EFV) and Tenofovir (TDF)/Emtricitabine. He took 18,000 mg of EFV (90 tablets) and 9,000 mg of TDF (30 tablets) with suicidal intent. At 24 h, plasma EFV concentration was 6,860 ng/mL, which was 8-fold higher compared with trough value (900 ng/mL) at single-dose administration. After 96 h, plasma EFV concentration decreased to 860 ng/mL. The elimination half-life of EFV was not prolonged in this case. In contrast, plasma TDF concentration was 310 ng/mL at 24 h and then increased to 370 ng/mL after 48 h. This value was 12-fold higher compared with trough value (30 ng/mL) at single-dose administration. After 96 h, plasma TDF concentration decreased to 30 ng/mL. This data suggests that the elimination half-life of TDF will be prolonged in overdose.

Conclusion : In this case, activated charcoal was available for adsorption of EFV. However, it was not suitable for adsorption of TDF, because TDF has a strong affinity for water. To excrete TDF from the body, an intravenous saline hydration was effective. A temporary increase of CK-MB may be caused by an injury of cardiac muscle.

Key words : ART, EFV, TDF, overdose

CASE REPORT

症例

免疫再構築症候群として発症したGraves病の1例*

古西　満　片浪雄一　忽那賢志
宇野健司　三笠桂一**

はじめに　抗HIV(human immunodeficiency virus)治療後にみられる免疫再構築症候群(immune reconstitution inflammatory syndrome：IRIS)の多くは日和見感染症である．しかし，ときには自己免疫疾患を発症することもあり，IRISは多彩な病状を呈する．

今回われわれは，サルベージ療法のため抗HIV治療を変更し，約26ヵ月後にIRISと考えるGraves病を発症した症例を経験したので報告する．

症　例

症　例：46歳，男性．

主　訴：動悸，下痢，体重減少．

家族歴：父は糖尿病，高血圧．姉は乳癌．

既往歴：1989年に慢性C型肝炎．

現病歴：血友病Aのため使用した非加熱血液製剤でHIVに感染し，1998年から抗HIV治療を開始した．仕事(長距離トラック)のため生活が不規則となり，服薬や受診が途切れがちであった．抗HIV薬の各クラスに耐性を認めたため，2004年ごろからCD4陽性細胞数が減少しはじめ，2007年7月には5/μlとなり，ニューモシスチス肺炎を発症した．ニューモシスチス肺炎の治療後9月初旬からsanilvudine(d4T)・darunavir(DRV)・ritonavir(RTV)・raltegravir(RAL)によるサルベージ療法を開始した．治療効果は良好で，HIV-RNA量は1ヵ月後には検出限界未満となり，CD4陽性細胞数も1年後には200/μl以上を維持するようになった．

その後病状は安定していたが，2009年11月中旬から食欲亢進，動悸，下痢，頻尿，体重減少が順次出現した．12月の外来受診時の検査で甲状腺機能亢進症の所見を認めたため，精査加療目的で12月下旬に入院した．

入院時身体所見：身長170 cm，体重63 kg，血圧120/78 mmHg，脈拍112/min・整，体温37.0℃．甲状腺は軽度腫大し，両側頸部に小豆大のリンパ節を数個触知した．両側膝関節の軽度変形を認めた．

入院時検査所見(Table 1)：軽度の貧血と肝機能障害を認め，CD4陽性細胞数は356/μl，HIV-RNA量は検出しなかった．TSHは低下し，FT3，FT4は増加していた．抗TPO抗体，抗TSH受容体抗体，甲状腺刺激型抗体は陽性であった．甲状腺シンチグラフィでヨード摂取率は59.4%と亢進していた．甲状腺エコーではびまん性に軽度腫大を認め，内部エコーは比較的均一であった．

臨床経過(Fig. 1)：2009年4月に行った甲状腺機能検査は正常値であったが，入院時には甲状腺機能亢進を認めた．また，抗TSH受容体抗体が陽性で，ヨード摂取率が亢進していたことから，Graves病と診断した．2008年9月の保存血清を用いて甲状腺関連の自己抗体を測定したが，すべて陰性であった．有効な抗HIV治療に変更して約26ヵ月後に発症したGraves病であり，IRISと考えた．

抗HIV治療は継続しながら，thiamazole(MMI)

---

* A Case of Graves' Disease as Immune Reconstitution Inflammatory Syndrome.

** M. Konishi(客員教授)，Y. Katanami, S. Kutsuna, K. Uno, K. Mikasa(教授)：奈良県立医科大学感染症センター．

Table 1. 入院時検査所見

末梢血		LDH	246 IU/l	HBs 抗原	(−)
WBC	5,200/μl	CK	58 IU/l	HCV 抗体	(+)
好中球	31 %	BUN	15 mg/dl	HCV-RNA	5.7 logIU/ml
好酸球	1 %	Cre	0.50 mg/dl	甲状腺関連	
リンパ球	49 %	T-cho	127 mg/dl	TSH	<0.03 μU/ml
単球	19 %	TG	172 mg/dl	FT3	19.4 pg/ml
RBC	397×10⁴/μl	Glu	99 mg/dl	FT4	6.04 ng/ml
Hb	12.8 g/dl	CRP	0.1 mg/dl	抗 Tg 抗体	≦0.3 U/ml
Ht	38.0 %	HIV 感染症関連		抗 TPO 抗体	8.5 U/ml
Plt	15.8×10⁴/μl	CD4⁺	356/μl	抗 TSH 受容体抗体	18.0 IU/l
赤沈	10 mm/1 hr	CD8⁺	796/μl	甲状腺刺激型抗体	350 %
生化学		HIV-RNA	検出せず	ヨード摂取率	59.4 %
AST	40 IU/l	肝炎ウイルス関連			
ALT	47 IU/l	HBs 抗体	(+)		

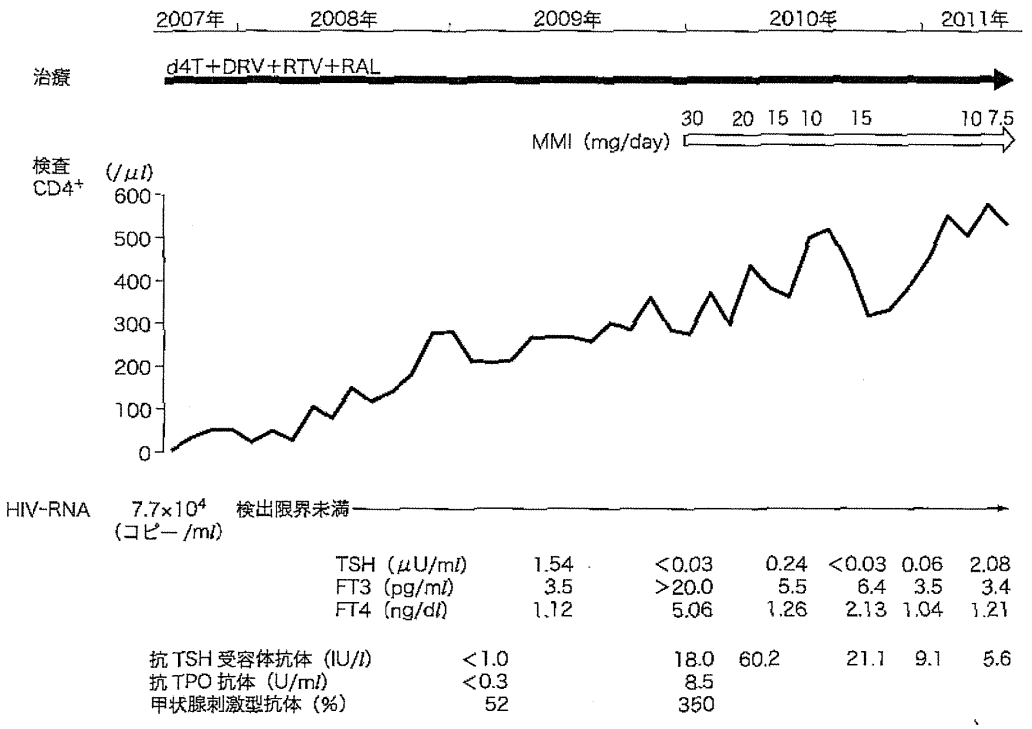

Fig. 1. 治療経過

30 mg/day の投与を開始した．約 2 週間で自覚症状は改善し，その後再発を認めていない．甲状腺機能をみながら MMI を減量し，現在は 7.5 mg/day となっている．抗 TSH 受容体抗体は MMI 投与後も 5ヵ月間は上昇したが，以後は減少し始め，2011 年 4 月には 5.6 IU/l となっている．

考　　察

免疫不全が進行した症例に有効な抗 HIV 治療を開始した後に，日和見感染症などが発症，再発，

再増悪することがある[1]．これは抗 HIV 治療によって HIV 複製の抑制と免疫能の改善が起こり，体内に存在する病原体などに対する免疫応答が強く誘導されるために生じる[2]と考えられている．そのため，この現象は IRIS と呼ばれている．IRIS の発症頻度は抗 HIV 治療例の 16.1(3～39)% と報告されている[3]．その病状の多くは日和見感染症であるが[4]，ときには日和見腫瘍や自己免疫疾患を発症することもある．

われわれの経験した症例は，甲状腺中毒症を示唆する動悸などの症状，血中甲状腺ホルモン濃度の上昇，抗 TSH 受容体抗体の陽性，甲状腺シンチグラフィでのヨード摂取率亢進を認めたので，Graves 病と診断した．さらに，高度の免疫不全状態で有効な抗 HIV 治療に変更した後に CD4 陽性細胞数が増加した状況で発病していることから，IRIS として発症した Graves 病と考えた．1998 年に Gilquin ら[5]が IRIS による Graves 病の 3 症例を報告し，最近同様の報告症例が増えている[6]．わが国でも，2003 年に守谷ら[7]がはじめて症例報告し，その後学会報告が散見されるようになっている[8]．

IRIS による日和見感染症は，抗 HIV 治療の開始・変更後 3ヵ月以内に発症することが多いが，Graves 病は大半が 1 年以上経過して発症すると指摘されている[6]．本症例も抗 HIV 治療を変更して 26ヵ月後に Graves 病を発症しており，ほかの症例報告と類似している．IRIS による Graves 病がなぜ抗 HIV 治療開始後 1 年以上を経過して発症するのかは，いまだ明確にはされていない．しかし，抗 HIV 治療後の CD4 陽性細胞の回復は，まずメモリー T 細胞が増加し始め，6ヵ月後プラトーに達した後に，ナイーブ T 細胞が増加し始めることが指摘されている．メモリー T 細胞の増加する時期には日和見感染症，ナイーブ T 細胞の増加する時期には自己免疫疾患が IRIS として発症すると推察されている[9]．本症例では，Graves 病発症前の保存血清の自己抗体は陰性であり，その後 14ヵ月間に自己抗体が形成されている．しかし，この 14ヵ月間で CD4 陽性細胞数は緩徐な増加がみられるのみで何が Graves 病発症の契機であるかを今後明らかにする必要がある．

わが国ではいまだに AIDS(acquired immunodeficiency syndrome)を発病してから医療機関を受診する症例が多い．しかし，抗 HIV 治療が進歩したので，高度な免疫不全状態であっても免疫状態を回復し，長期生存が可能となっている．そのため，HIV 感染者の治療経過中に IRIS による Graves 病を経験することが増えてくる可能性があり，そのことに留意して診療する必要があると考える．

文　献

1) French MA et al：Zidovudine-induced restoration of cell-mediated immunity to mycobacteria in immunedeficient HIV-infected patients. AIDS 6：1293, 1992
2) Lipman M, Breen R.：Immune reconstitution inflammatory syndrome in HIV. Curr Opin Infect Dis 19：20, 2006
3) Müller M et al：Immune reconstitution inflammatory syndrome in patients starting antiretroviral therapy for HIV infection：a systematic review and meta-analysis. Lancet Infect Dis 10：251, 2010
4) 古西　満ほか：免疫再構築症候群の発症状況調査．厚生労働科学研究費補助金エイズ対策研究事業 HAART 時代の日和見合併症に関する研究 平成 15 年度総括・分担研究報告書，82 頁，2004
5) Gilquin J et al：Delayed occurrence of Graves' disease after immune restoration with HAART. Lancet 352：1907, 1998
6) Crum NF et al：Graves disease：an increasingly recognized immune reconstitution syndrome. AIDS 20：466, 2006
7) 守谷研二ほか：HAART 中の Graves 病を発症した HIV 感染症例．日エイズ会誌 5：158，2003
8) 濱田佳寿ほか：HAART 療法中にバセドウ病を発症した一例．日内分泌会誌 86：286，2010
9) Chen F：Characteristics of autoimmune thyroid disease occurring as a late complication of immune reconstitution in patients with advanced human immunodeficiency virus(HIV) disease. Medicine 84：98, 2005

JCM
Journals.ASM.org

Clinical and Microbiological Differences between *Mycobacterium abscessus* and *Mycobacterium massiliense* Lung Diseases

Toshiyuki Harada,[a] Yasushi Akiyama,[a] Atsuyuki Kurashima,[b] Hideaki Nagai,[c] Kazunari Tsuyuguchi,[d] Takashi Fujii,[e] Syuichi Yano,[f] Eriko Shigeto,[g] Toshihiko Kuraoka,[h] Akira Kajiki,[i] Yoshihiro Kobashi,[j] Fumio Kokubu,[k] Atsuo Sato,[l] Shiomi Yoshida,[m] Tomotada Iwamoto,[n] and Hajime Saito[o]

Center for Respiratory Diseases, Hokkaido Social Insurance Hospital, Sapporo, Japan[a]; Clinical Research Advisor, Japan Anti-Tuberculosis Association, Fukujuji Hospital, Kiyose, Japan[b]; Center for Respiratory Diseases, National Hospital Organization Tokyo National Hospital, Kiyose, Japan[c]; Department of Respiratory Medicine, Clinical Research Center, National Hospital Organization Kinki-Chuo Chest Medical Center, Sakai, Japan[d]; Department of Internal Medicine, Japan Anti-Tuberculosis Association Osaka Branch, Osaka Hospital, Neyagawa, Japan[e]; Department of Pulmonary Medicine, National Hospital Organization Matsue Medical Center, Matsue, Japan[f]; Department of Respiratory Medicine, National Hospital Organization Higashihiroshima Medical Center, Higashihiroshima, Japan[g]; Internal Medicine, Kyosai-Yoshijima Hospital, Hiroshima, Japan[h]; Department of Respiratory Medicine, National Hospital Organization Omuta Hospital, Omuta, Japan[i]; Division of Respiratory Diseases, Department of Medicine, Kawasaki Medical School, Kurashiki, Japan[j]; Department of Respiratory Medicine, Showa University Fujigaoka Hospital, Yokohama, Japan[k]; Respiratory Department, National Hospital Organization Minami-Kyoto Hospital, Jouyou, Japan[l]; Clinical Research Center, National Hospital Organization Kinki-Chuo Chest Medical Centre, Sakai, Japan[m]; Department of Microbiology, Kobe Institute of Health, Kobe, Japan[n]; and Hiroshima Environment & Health Association, Health Science Center, Hiroshima, Japan[o]

In recent years, many novel nontuberculous mycobacterial species have been discovered through genetic analysis. *Mycobacterium massiliense* and *M. bolletii* have recently been identified as species separate from *M. abscessus*. However, little is known regarding their clinical and microbiological differences in Japan. We performed a molecular identification of stored *M. abscessus* clinical isolates for further identification. We compared clinical characteristics, radiological findings, microbiological findings, and treatment outcomes among patients with *M. abscessus* and *M. massiliense* lung diseases. An analysis of 102 previous isolates of *M. abscessus* identified 72 (71%) *M. abscessus*, 27 (26%) *M. massiliense*, and 3 (3%) *M. bolletii* isolates. Clinical and radiological findings were indistinguishable between the *M. abscessus* and *M. massiliense* groups. Forty-two (58%) patients with *M. abscessus* and 20 (74%) patients with *M. massiliense* infections received antimicrobial treatment. Both the *M. abscessus* and *M. massiliense* groups showed a high level of resistance to all antimicrobials, except for clarithromycin, kanamycin, and amikacin. However, resistance to clarithromycin was more frequently observed in the *M. abscessus* than in the *M. massiliense* group (16% and 4%, respectively; $P = 0.145$). Moreover, the level of resistance to imipenem was significantly lower in *M. abscessus* isolates than in *M. massiliense* isolates (19% and 48%, respectively; $P = 0.007$). The proportions of radiological improvement, sputum smear conversion to negativity, and negative culture conversion during the follow-up period were higher in patients with *M. massiliense* infections than in those with *M. abscessus* infections. Patients with *M. massiliense* infections responded more favorably to antimicrobial therapy than those with *M. abscessus* infections.

Mycobacterium species are common causes of pulmonary infections in both humans and animals (14). Although members of the *Mycobacterium tuberculosis* complex cause the majority of pulmonary infections worldwide, many nontuberculous mycobacteria (NTM) can cause similar infections (13, 20). In recent years, many novel NTM species have been discovered through the increased application of genetic investigation tools; detailed genetic characterizations have helped define new taxonomic groupings (17, 29). Recently, two new *M. abscessus*-related species, *M. massiliense* and *M. bolletii*, were identified, which were previously grouped with *M. abscessus* (1, 3). The rate of isolation of these two species has been increasing in Japan. However, very little is known about the natural epidemiology and pathogenicity of *M. massiliense* and *M. bolletii* outside outbreak situations. One report found that the ratio of *M. abscessus* to all NTM is much higher in South Korea (19) than in other countries, including Japan.

Here, we aimed to evaluate the epidemiology, clinical and radiological spectrum, treatments, drug susceptibility, and outcome of *M. abscessus* and *M. massiliense* lung diseases during therapy in Japan.

MATERIALS AND METHODS

Study population. We retrospectively reviewed the medical records of patients initially diagnosed with *M. abscessus* lung disease according to the 2007 American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) guidelines (16) between January 1990 and December 2010 at 12 hospitals or institutions in Japan. These *M. abscessus* species were thereafter identified as *M. abscessus*, *M. massiliense*, and *M. bolletii*. Clinical, radiological, microbiological, management, and outcome data were collected from medical files. Permission was obtained from the institutional review board committee of Hokkaido Social Insurance Hospital (approval number 2011-11). Informed consent was waived because of the retrospective nature of the study.

Received 9 May 2012 Returned for modification 24 July 2012
Accepted 14 August 2012
Published ahead of print 22 August 2012
Address correspondence to Toshiyuki Harada, t-harada@hok-shaho-hsp.jp.
Supplemental material for this article may be found at http://jcm.asm.org/.

doi:10.1128/JCM.01175-12

Microbiological examination. Sputum or bronchoalveolar lavage fluid was used for smears and mycobacterial cultures according to standard methods (5). Any processed specimens that remained were stored at 2°C to 8°C for the duration of culturing in the study to allow the retesting of the specimens that showed a discrepancy in results between culture growth and preliminary identification by a DNA-DNA hybridization (DDH) mycobacterium kit (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan). Samples were cultured by using the Bactec MGIT 960 system or Ogawa solid medium, and the isolates were stored at −30°C to −80°C. *M. abscessus* species were preliminarily identified by microplate DDH technology using the DDH mycobacterium kit (21) at each hospital or institution. We collected all frozen isolates for multisequencing and susceptibility testing. Frozen isolates were recultured by using the MGIT 960 system and Ogawa solid medium and checked for contamination by growth on *p*-nitrobenzoic acid agar medium.

Further differentiation among *M. abscessus* species was performed at the Department of Mycobacteriology, Leprosy Research Centre, National Institute of Infectious Disease, and the Kobe Institute of Health. Sequences of clinical isolates which were previously identified as *M. abscessus* by DDH were compared with the reference *M. abscessus* (JCM 15300^T), *M. massiliense* (JCM 13569^T), and *M. bolletii* (JCM 15297^T) strains. The majority of the 16S rRNA gene, partial aspects of the *hsp65* and *rpoB* genes, and the 16S-23S rRNA internal transcribed spacer (ITS) region were amplified by PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and primers described previously (22). The PCR products were sequenced with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI Prism 310 genetic analyzer (Applied Biosystems). Sequences were analyzed for their similarity to sequences in the GenBank database by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Antimycobacterial susceptibility testing was performed at the National Hospital Organization Kinki-Chuo Chest Medical Center (Osaka, Japan). Susceptibility was determined by MIC methods using the Etest and broth microdilution.

Etest. Isolates of rapidly growing mycobacteria (RGM) were mailed on Ogawa slant medium and subcultured on 5% sheep blood agar at 35°C in ambient air for 72 h. Bacterial suspensions were prepared in cation-adjusted Mueller-Hinton broth (Eiken Chemical, Tokyo, Japan) to a 1.0 McFarland standard and plated onto cation-adjusted Mueller-Hinton agar. Etest strips (Sysmex bioMérieux, Tokyo, Japan) were placed onto Mueller-Hinton agar (Becton Dickinson, Fukushima, Japan) according to the manufacturer's instructions, and the results were read after 72 h of incubation. Final concentration ranges were 0.016 to 256 μg/ml for isoniazid (INH) and ethambutol (EB); 0.002 to 32 μg/ml for rifampin (RFP), ciprofloxacin (CPFX), and moxifloxacin (MFLX); and 0.064 to 1,024 μg/ml for streptomycin (SM). Susceptibility was evaluated according to Clinical and Laboratory Standards Institute (CLSI) breakpoint recommendations (8, 37) and those proposed previously by Woods et al. (36), Wallace et al. (33), Yang et al. (38), and Swenson et al. (27, 28).

Broth microdilution. Serial double dilutions of clarithromycin (CAM), kanamycin (KM), amikacin (AMK), and imipenem (IPM) were prepared at a concentration range of 0.015 to 64 μg/ml according to CLSI recommendations (37). Briefly, pure colonies were cultured in 7H9 broth with 0.2% glycerol in a tube for 3 to 5 days, vigorously vortexed, and then adjusted to a density equivalent of 0.5 on the McFarland scale. Bacterial suspensions in cation-adjusted Mueller-Hinton broth were transferred into the wells of dry microdilution plates containing the antimicrobial agents (Eiken Chemical). The inoculated plates were placed into plastic bags, incubated at 35°C in ambient air, and read after 72 h. The MIC was defined as the lowest concentration of drug that inhibited visible growth. Susceptibility was evaluated according to CLSI breakpoint recommendations (8, 37) and those proposed previously by Shen et al. (24).

Data analysis. The results are expressed as ranges and means or as numbers of patients. Categorical variables were analyzed by using the χ^2 or Fisher's exact test. Continuous variables were analyzed by using the Mann-Whitney U test or *t* test. All *P* values are two sided; a *P* value of <0.05 indicates statistical significance. Statistical analyses were performed by using Excel 2011 (Microsoft) with the add-in software Statcel 3 (OMS Publishing Inc., Saitama, Japan).

RESULTS

Identification of clinical isolates. Clinical isolates from 102 patients previously diagnosed with *M. abscessus* lung disease by DDH were identified by sequence analysis of the 16S rRNA, *hsp65*, *rpoB*, and ITS genes. Seventy-two (71%) isolates were identified as *M. abscessus*, 27 (26%) were identified as *M. massiliense*, and 3 (3%) were identified as *M. bolletii*.

Patient characteristics. We compared clinical characteristics and treatment outcomes among the 72 patients with *M. abscessus* and 27 patients with *M. massiliense* infections. Patients with *M. bolletii* infection were not included for further study because of their small number.

Table 1 summarizes the baseline characteristics of the patients. No significant differences were found between the *M. abscessus* and *M. massiliense* groups in any of the baseline characteristics, including demographic data, underlying conditions, and respiratory symptoms. The distributions of radiographic disease types were similar in both groups, except for bronchiectasis. Bronchiectasis was significantly more frequent in the *M. abscessus* group (73%; 40 of 55) than in the *M. massiliense* group (43%; 10 of 23) ($P = 0.014$). None of these patients tested positive for HIV.

Drug susceptibility. Table 2 shows the drug susceptibility results for 63 *M. abscessus* and 23 *M. massiliense* isolates, and Table S1 in the supplemental material shows the MIC distributions of the strains. The drug was determined to be effective against an isolate if the MIC of the antimicrobial agent was less than the susceptible concentration, as shown in Table 2. Of the parenteral antibiotics, KM and AMK were effective against most *M. abscessus* isolates (95% and 94%, respectively) and *M. massiliense* isolates (100% for both), with no difference between the species ($P = 0.388$ and 0.509, respectively). However, SM was ineffective against both *M. abscessus* (67%) and *M. massiliense* (61%) isolates ($P = 0.617$). Moreover, the rates of IPM drug resistance were significantly higher in *M. massiliense* than in *M. abscessus* isolates (48% and 19%, respectively; $P = 0.007$).

Of the oral antibiotics, CAM was effective against most *M. abscessus* and *M. massiliense* isolates (84% and 96%, respectively; $P = 0.145$). In contrast, MFLX, CPFX, and RFP were ineffective against most *M. abscessus* (92%, 95%, and 97%, respectively) and *M. massiliense* (96%, 91%, and 100%, respectively) isolates, with no difference between the species ($P = 0.488$, 0.883, and 0.534, respectively). We could not interpret the INH and EB susceptibilities because no breakpoints have been established for these drugs.

Antimicrobial treatment and response. Of the 102 patients, 42 (58%) with *M. abscessus*, 20 (74%) with *M. massiliense*, and 2 (67%) with *M. bolletii* infections received antimicrobial treatment for 3 to 178 months (mean = 33 months), 1 to 122 months (mean = 36 months), and 4 to 68 months (mean = 36 months), respectively. Forty-two patients with *M. abscessus* lung disease and 20 patients with *M. massiliense* lung disease were analyzed for treatment response (Table 3). Radiographic improvement rates were lower for patients with *M. abscessus* infection than for those with *M. massiliense* infection (29% and 48%, respectively; $P = 0.101$).

Microbiological responses also differed between the two

TABLE 1 Clinical characteristics of patients with *Mycobacterium abscessus* and *M. massiliense* lung disease

Characteristic[a]	Value for group		*P* value
	M. abscessus ($n = 72$)	*M. massiliense* ($n = 27$)	
Age (yr) [range (mean)]	27–94 (68)	44–84 (67)	0.637
No. of males/no. of females	26/45	13/13	0.233
Mean body mass index (kg/m^2)	19.5	18.8	0.699
No. of patients with symptom			
Cough	25	9	0.931
Sputum	22	9	0.759
Hemoptysis	14	8	0.262
Fever	8	5	0.905
Dyspnea	4	5	0.057
No. of patients with underlying disease			
Previous pulmonary tuberculosis	12	7	0.297
NTM (MAC)	15	4	0.516
Mycosis	4	2	0.798
Interstitial pneumonia	4	2	0.803
COPD	3	2	0.879
Diabetes mellitus	3	3	0.196
Steroid use	3	3	0.196
Malignancy	2	1	0.823
No. of smokers/no. of nonsmokers	33/11	12/8	0.223
No. of patients who consumed alcohol/no. of patients who did not consume alcohol	30/6	11/3	0.490
No. of patients with/no. of patients without radiological finding of:			
Cavitation	28/28	14/9	0.379
Centrilobular lesion	38/19	14/11	0.355
Infiltration	35/16	12/11	0.173
Bronchiectasis	40/15	10/13	0.014
Pleural effusion	2/49	2/22	0.383
No. of patients with positive AFB smear	50	21	0.363

[a] Abbreviations: NTM, nontuberculous mycobacteria; MAC, *Mycobacterium avium* complex; COPD, chronic obstructive pulmonary disease; AFB, acid-fast bacillus.

groups. The initial sputum conversion rates were lower in patients with *M. abscessus* infection than in those with *M. massiliense* infection (31% and 50%, respectively; $P = 0.115$). The sputum acid-fast bacillus (AFB)-positive relapse rate after the initial conversion to a negative result was higher for patients with *M. abscessus* infection than for those with *M. massiliense* infection (65% and 30%, respectively; $P = 0.077$). Thus, the proportion of patients whose sputum converted and remained culture negative during the follow-up period was lower for patients with *M. abscessus* infection than for those with *M. massiliense* infection.

DISCUSSION

The *M. abscessus* group comprises ubiquitous environmental organisms frequently associated with nosocomial outbreaks and pseudo-outbreaks (18, 32, 34). The increasing availability of gene sequencing has tremendously influenced the taxonomy of bacteria, particularly mycobacteria, with many new species being described every year (30).

A new species related to *M. abscessus*, *M. massiliense*, was recently described (3). The species *M. massiliense* was proposed in 2004 based upon nonconventional phenotypic characterization and genotypic studies of 2 isolates recovered from the sputum and bronchoalveolar fluid of a patient in France (3). Since *M. massiliense* is closely related to *M. abscessus*, it is possible that *M. massiliense* infections have overlapped with *M. abscessus* infections in previous reports (3). Although several unique phenotypes that differentiate *M. massiliense* from *M. abscessus* have been identified, they are difficult to characterize using conventional clinical techniques because of the inability to discriminate between RGM due to their overlapping phenotypic patterns (9, 26). The molecular, biological, and clinical characteristics of these strains will help us to better understand and treat severe infections due to RGM (39). The proper identification of members of the *M. abscessus* complex has proven beneficial in both therapeutic management and epidemiological studies. *M. massiliense* is very closely related to *M. abscessus* but showed different susceptibilities to CAM, and their pathogenic potentials have been demonstrated by infections of immunocompetent and immunocompromised hosts (15, 18).

The proportions of *M. massiliense* strains among *M. abscessus* species vary according to geographical distribution. The prevalences of *M. massiliense* were 28% of 40 patients at the National Institutes of Health in the United States (39), 21% of 39 clinical isolates in the Netherlands (31), 22% of 50 patients with cystic fibrosis in France (23), 55% of 150 patients in South Korea (19), and 26% of 102 patients in Japan. There is currently no explanation for the large difference in the prevalences of *M. massiliense* between South Korea and Japan, as they are in the same Asian region.

No significant differences were found between the baseline clinical characteristics in the *M. abscessus* and *M. massiliense* groups, except for bronchiectasis in the radiological findings. Bronchiectasis was found significantly more frequently in the *M. abscessus* group than in the *M. massiliense* group (73% and 43%, respectively; $P = 0.014$). This result is almost consistent with the results of a Korean study (19).

Importantly, favorable microbiological response rates with similar combinations of antibiotic therapy were much higher for *M. massiliense* than for *M. abscessus* lung disease. This could be explained by the differences in CAM resistance. This study demonstrated a high level of resistance to CAM in *M. abscessus* isolates but not in *M. massiliense* isolates, indicating that treatment of *M. abscessus* lung disease may be more difficult. In fact, *M. abscessus* lung disease has been regarded as a chronic, incurable infection for most patients, given the current antibiotic options (4). The low MIC and absence of CAM resistance (except for one isolate) suggest that *M. massiliense* lung disease may be treated more effectively with a CAM-based antibiotic regimen. Recent studies showed that some RGM, such as *M. abscessus* and *M. fortuitum*, have an *erm* gene that induces macrolide resistance (4, 12). It is

TABLE 2 *In vitro* susceptibilities of 63 *Mycobacterium abscessus* and 23 *M. massiliense* isolates to different antimicrobials[a]

Drug	Species	MIC (μg/ml) for categorization of susceptibility of:			Reference(s)	% resistant isolates (no. of resistant isolates)	*P* value
		Susceptible	Intermediate	Resistant			
Clarithromycin	*M. abscessus*	≤2	4	≥8	15	16 (10)	0.145
	M. massiliense					4 (1)	
Kanamycin[b]	*M. abscessus*	≤16	32	≥64	14, 20	5 (3)	0.388
	M. massiliense					0 (0)	
Amikacin	*M. abscessus*	≤16	32	≥64	15	6 (4)	0.509
	M. massiliense					0 (0)	
Imipenem	*M. abscessus*	≤4	8–16	≥32	15	19 (12)	0.007
	M. massiliense					48 (11)	
Moxifloxacin	*M. abscessus*	≤1	2	≥4	15	92 (58)	0.488
	M. massiliense					96 (22)	
Ciprofloxacin	*M. abscessus*	≤1	2	≥4	15	95 (60)	0.883
	M. massiliense					91 (21)	
Isoniazid[c]	*M. abscessus*	NA	NA	NA		NA	
	M. massiliense					NA	
Rifampin[b]	*M. abscessus*	≤1	2	≥4	14, 18	97 (61)	0.534
	M. massiliense					100 (23)	
Ethambutol[c]	*M. abscessus*	NA	NA	NA		NA	
	M. massiliense					NA	
Streptomycin[b]	*M. abscessus*	≤32	NA	≥64	14, 20	67 (42)	0.617
	M. massiliense					61 (14)	

[a] Drug susceptibility results are shown for 63 patients with *M. abscessus* and 23 patients with *M. massiliense* infections. NA, not available.
[b] The CLSI breakpoints (8) for *Staphylococcus* species have been substituted as the breakpoints of these drugs against *M. abscessus* and *M. massiliense* isolates.
[c] The breakpoints for *M. abscessus* and *M. massiliense* isolates have not yet been established.

unknown whether other RGM such as *M. massiliense* have an *erm* gene. Thus, species-level identification is important because antibiotic susceptibilities and therapies differ significantly depending on the RGM species (4, 16). Since the KM and AMK resistance rates were less than 10% for both the *M. abscessus* and *M. massiliense* groups in the present study, KM and AMK could be two key drugs for the treatment of *M. abscessus* and *M. massiliense* lung diseases.

The IPM resistance rate was much lower in the present study than in the Korean study (19) (19% and 44% for *M. abscessus* and 48% and 67% for *M. massiliense*, respectively). The MFLX, CPFX, and RFP resistance rates were >90% for both the *M. abscessus* and *M. massiliense* groups in the present study. These findings are compatible with the fact that the *M. abscessus* complex has been regarded as being fluoroquinolone resistant (7). However, moderate *in vitro* activities of some fluoroquinolones against members of the *M. abscessus* complex have been demonstrated (2, 6, 19, 25). The use of fluoroquinolones as alternative oral agents during combination antibiotic therapy for *M. abscessus* and *M. massiliense* infections should be studied further.

The present study showed poor activities of INH and EB in both the *M. abscessus* and *M. massiliense* groups (MIC_{90} for INH of ≥512 μg/ml; MIC_{90} for EB of ≥32 μg/ml), which are identical to findings reported previously (27). Thus, INH and EB seem to be ineffective against both the *M. abscessus* and *M. massiliense* groups. Another study found that MFLX was active against *M. abscessus* and that a combination of CAM and MFLX was effective against *M. abscessus* strains in *in vitro* models (10). Moreover, some *M. abscessus* isolates are susceptible to the oral drug linezolid (35, 38). However, linezolid was rarely used in our survey to treat *M. abscessus* species lung disease because of the high cost and moderate to severe side effects in Japan. Thus, further studies are required to evaluate active combinations of oral antibiotics and determine their clinical significance.

The present study has several limitations. First, the retrospective study design necessitates the use of medical records for data collection, leading to variations in each factor. Second, the number of sputum specimens collected over time was relatively small. Had samples been collected more frequently, more conversions to negativity and relapses after conversion may have been found. Third, treatment regimens cannot be optimized based solely on retrospective studies with limited follow-up data. Moreover, the use of IPM, cefoxitin, fluoroquinolone, and linezolid is not permitted for the treatment of NTM diseases under the Japanese social health insurance system. Thus, the combination therapy recommended by the ATS/IDSA (16) has not been applied in most cases. Treatment regimens were decided in practice by physicians. Therefore, it is difficult to evaluate the true treatment response in this study.

In conclusion, we found clinically significant differences be-

TABLE 3 Antimicrobial treatments and treatment responses

Characteristic[a]	Value for group		P value
	M. abscessus ($n = 72$)	*M. massiliense* ($n = 27$)	
Treatment			
No. of patients with/no. of patients without operation	3/69	3/24	0.201
No. of patients receiving/no. of patients not receiving chemotherapy	42/30	20/7	0.149
No. of patients on chemotherapy regimen of:			
M, R, E	8	5	
M, C, A	6	5	
M	8	0	
M, F	6	0	
H, R, E	2	2	
Other	12	8	
Duration of treatment (mo) [range (mean)]	3–178 (33)	1–122 (36)	0.723
Result			
No. of patients with/no. of patients without radiological improvement	17/41	12/13	0.101
No. of patients with/no. of patients without sputum smear conversion to negativity	17/38	11/11	0.115
No. of patients with relapse/no. of patients without relapse after sputum smear conversion to negativity	13/7	3/7	0.077
Duration of positive sputum results (mo) [range (mean)]	1–120 (25)	1–62 (18)	0.776

[a] Abbreviations: M, macrolides (clarithromycin, erythromycin, and azithromycin); A, aminoglycosides (streptomycin, amikacin, and kanamycin); F, fluoroquinolones (levofloxacin, moxifloxacin, garenoxacin, and gatifloxacin); C, carbapenems (imipenem and meropenem); H, isoniazid; R, rifampin; E, ethambutol.

tween *M. abscessus* and *M. massiliense* lung infections in Japan. Treatment responses rates with CAM-based antibiotic therapy were higher for *M. massiliense* than in *M. abscessus* lung disease. This difference in treatment responses may be explained by the difference in CAM susceptibilities between the two groups. Prospective clinical trials are needed to clarify these aspects.

ACKNOWLEDGMENTS

We thank Kazue Nakanaga, Department of Mycobacteriology, Leprosy Research Centre, National Institute of Infectious Diseases, for providing cultures. Angela Morben, a medical copy editor, assisted in the preparation and editing of the manuscript. Primary data and analysis results were made available to all coauthors.

Data analysis of reviewed medical records was performed at the Hokkaido Social Insurance Hospital. The identification of *M. abscessus* species by microplate DDH technology was performed at each hospital or institution. Further differentiation among *M. abscessus* species was performed at the Leprosy Research Center, National Institute of Infectious Diseases, and the Kobe Institute of Health. Antimycobacterial susceptibility testing was performed at the National Hospital Organization Kinki-Chuo Chest Medical Center.

We have no potential conflicts of interest to report. All authors have submitted a International Committee of Medical Journal Editors form for disclosure of potential conflicts of interest.

REFERENCES

1. **Adékambi T, Berger P, Raoult D, Drancourt M.** 2006. *rpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. Int. J. Syst. Evol. Microbiol. **56**:133–143.
2. **Adékambi T, Colson P, Drancourt M.** 2003. *rpoB*-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. J. Clin. Microbiol. **41**:5699–5708.
3. **Adékambi T, et al.** 2004. Amoebal coculture of "*Mycobacterium massiliense*" sp. nov. from the sputum of a patient with hemoptoic pneumonia. J. Clin. Microbiol. **42**:5493–5501.
4. **American Thoracic Society.** 1997. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. Am. J. Respir. Crit. Care Med. **156**(2 Pt 2):S1–S25.
5. **American Thoracic Society.** 2000. Diagnostic standards and classification of tuberculosis in adults and children. This official statement of the American Thoracic Society and the Centers for Disease Control and Prevention was adopted by the ATS Board of Directors, July 1999. This statement was endorsed by the Council of the Infectious Disease Society of America, September 1999. Am. J. Respir. Crit. Care Med. **161**(4 Pt 1):1376–1395.
6. **Blackwood KS, et al.** 2000. Evaluation of *recA* sequences for identification of *Mycobacterium* species. J. Clin. Microbiol. **38**:2846–2852.
7. **Chadha R, Grover M, Sharma A.** 1998. An outbreak of post-surgical wound infections due to *Mycobacterium abscessus*. Pediatr. Surg. Int. **13**: 406–410.
8. **Cockerill FR, III, et al.** 2011. Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement, 1st ed. M100-S21. Clinical and Laboratory Standards Institute, Wayne, PA.
9. **Conville PS, Witebsky FG.** 1998. Variables affecting results of sodium chloride tolerance test for identification of rapidly growing mycobacteria. J. Clin. Microbiol. **36**:1555–1559.
10. **Cremades R, et al.** 2009. Screening for sterilizing activity of antibiotic combinations in an acid model of rapidly growing mycobacteria during the stationary phase of growth. Chemotherapy **55**:114–118.
11. **Dauendorffer JN, et al.** 2003. Identification of mycobacterial species by PCR sequencing of quinolone resistance-determining regions of DNA gyrase genes. J. Clin. Microbiol. **41**:1311–1315.
12. **De Groote MA, Huitt G.** 2006. Infections due to rapidly growing mycobacteria. Clin. Infect. Dis. **42**:1756–1763.
13. **El-Solh AA, et al.** 1998. Clinical and radiographic manifestations of uncommon pulmonary nontuberculous mycobacterial disease in AIDS patients. Chest **114**:138–145.
14. **Falkinham JO, III.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. **9**:177–215.
15. **Freudenberger RS, Simafranca SM.** 2006. Cutaneous infection with rapidly-growing mycobacterial infection following heart transplant: a case report and review of the literature. Transplant. Proc. **38**:1526–1529.
16. **Griffith DE, et al.** 2007. An official ATS/IDSA statement. Diagnosis, treatment, and prevention of nontuberculous mycobacterial disease: Joint Tuberculosis Committee guidelines. Am. J. Respir. Crit. Care Med. **175**: 367–416.
17. **Hanna BA, et al.** 1999. Multicenter evaluation of the BACTEC MGIT 960 system for recovery of mycobacteria. J. Clin. Microbiol. **37**:748–752.
18. **Kim HY, et al.** 2007. Outbreak of *Mycobacterium massiliense* infection

associated with intramuscular injections. J. Clin. Microbiol. 45:3127–3130.
19. **Koh WJ, et al.** 2011. Clinical significance of differentiation of *Mycobacterium massiliense* from *Mycobacterium abscessus*. Am. J. Respir. Crit. Care Med. 183:405–410.
20. **Koh WJ, Kwon OJ, Lee KS.** 2002. Nontuberculous mycobacterial pulmonary diseases in immunocompetent patients. Kor. J. Radiol. 3:145–157.
21. **Kusunoki S, et al.** 1991. Application of colorimetric microdilution plate hybridization for rapid genetic identification of 22 *Mycobacterium* species. J. Clin. Microbiol. 29:1596–1603.
22. **Nakanaga K, et al.** 2011. Multiple cases of cutaneous *Mycobacterium massiliense* infection in a "hot spa" in Japan. J. Clin. Microbiol. 49:613–617.
23. **Roux AL, et al.** 2009. Multicenter study of prevalence of nontuberculous mycobacteria in patients with cystic fibrosis in France. J. Clin. Microbiol. 47:4124–4128.
24. **Shen GH, Wu BD, Wu KM, Chen JH.** 2007. In vitro activities of isepamicin, other aminoglycosides, and capreomycin against clinical isolates of rapidly growing mycobacteria in Taiwan. Antimicrob. Agents Chemother. 51:1849–1851.
25. **Simmon KE, et al.** 2007. Identification of an emerging pathogen, *Mycobacterium massiliense*, by *rpoB* sequencing of clinical isolates collected in the United States. J. Clin. Microbiol. 45:1978–1980.
26. **Springer B, Stockman L, Teschner K, Roberts GD, Böttger EC.** 1996. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J. Clin. Microbiol. 34:296–303.
27. **Swenson JM, Thornsberry C, Silcox VA.** 1982. Rapidly growing mycobacteria: testing of susceptibility to 34 antimicrobial agents by broth microdilution. Antimicrob. Agents Chemother. 22:186–192.
28. **Swenson JM, Wallace RJ, Jr, Silcox VA, Thornsberry C.** 1985. Antimicrobial susceptibility of five subgroups of *Mycobacterium fortuitum* and *Mycobacterium chelonae*. Antimicrob. Agents Chemother. 28:807–811.
29. **Tortoli E.** 2003. Impact of genetic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin. Microbiol. Rev. 16:319–354.
30. **Tortoli E.** 2006. The new mycobacteria: an update. FEMS Immunol. Med. Microbiol. 48:159–178.
31. **van Ingen J, de Zwaan R, Dekhuijzen RP, Boeree MJ, van Soolingen D.** 2009. Clinical relevance of *mycobacterium chelonae-abscessus* group isolation in 95 patients. J. Infect. 59:324–331.
32. **Viana-Niero C, et al.** 2008. Molecular characterization of *Mycobacterium massiliense* and *Mycobacterium bolletii* in outbreaks of infections after laparoscopic surgeries and cosmetic procedures. J. Clin. Microbiol. 46: 850–855.
33. **Wallace RJ, Jr, Brown-Elliott BA, Crist CJ, Mann L, Wilson RW.** 2002. Comparison of the in vitro activity of the glycylcycline tigecycline (formerly GAR-936) with those of tetracycline, minocycline, and doxycycline against isolates of nontuberculous mycobacteria. Antimicrob. Agents Chemother. 46:3164–3167.
34. **Wallace RJ, Jr, Brown BA, Griffith DE.** 1998. Nosocomial outbreaks/pseudo-outbreaks caused by nontuberculous mycobacteria. Annu. Rev. Microbiol. 52:453–490.
35. **Wallace RJ, Jr, et al.** 2001. Activities of linezolid against rapidly growing mycobacteria. Antimicrob. Agents Chemother. 45:764–767.
36. **Woods GL, et al.** 2000. Multisite reproducibility of Etest for susceptibility testing of *Mycobacterium abscessus*, *Mycobacterium chelonae*, and *Mycobacterium fortuitum*. J. Clin. Microbiol. 38:656–661.
37. **Woods GL, et al.** 2011. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes. Approved standard, 2nd ed. M24-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
38. **Yang SC, et al.** 2003. High prevalence of antimicrobial resistance in rapidly growing mycobacteria in Taiwan. Antimicrob. Agents Chemother. 47:1958–1962.
39. **Zelanzy AM, et al.** 2009. Cohort study of molecular identification and typing of *Mycobacterium abscessus*, *Mycobacterium massiliense*, and *Mycobacterium bolletii*. J. Clin. Microbiol. 47:1985–1995.

INTERNAL MEDICINE

□ CASE REPORT □

A Patient with Relapsing Polychondritis who Had Been Diagnosed as Intractable Bronchial Asthma

Ryota Sato[1], Nobuharu Ohshima[2], Kimihiko Masuda[1], Hirotoshi Matsui[1], Naoko Higaki[1], Eri Inoue[1], Jun Suzuki[1], Hideaki Nagai[1], Shinobu Akagawa[1], Akira Hebisawa[3] and Shunsuke Shoji[2]

Abstract

A 62-year-old woman, diagnosed as bronchial asthma 3 years previously, was admitted due to acute severe dyspnea. Physical examination revealed saddle nose, flare/swelling of the ear auricles, and stridor. Computed tomography demonstrated thickening of tracheal/bronchial walls and stenosis of the lumen that deteriorated on expiration, suggesting tracheobronchomalacia. Auricle biopsy indicated cartilage destruction. Based on these findings, the patient was diagnosed as relapsing polychondritis. As demonstrated in this case, relapsing polychondritis involving airways might be misdiagnosed as bronchial asthma due to stridor and transient corticosteroid-related improvement. Early diagnosis is necessary to prevent irreversible airway stenosis and progression to tracheobronchomalacia.

Key words: intractable bronchial asthma, saddle nose, takotsubo cardiomyopathy, tracheobronchomalacia, relapsing polychondritis

(Intern Med 51: 1773-1778, 2012)
(DOI: 10.2169/internalmedicine.51.7621)

Introduction

Relapsing polychondritis (RPC), considered to be an autoimmune disease (1), involves general cartilage and tissues containing a high concentration of mucopolysaccharides. This is a rare disorder with an estimated incidence of 3.5/1,000,000 persons/year (2), and the treatment has not been established. When tracheal/bronchial cartilages are affected, respiratory symptoms such as dyspnea and stridor may appear (3), which can be misleading, prompting an improper diagnosis as bronchial asthma (4). It could take long until correct diagnosis was made (5). Here, we report a patient with RPC who had been diagnosed as intractable bronchial asthma for a long period of time. Since RPC could be fatal, it is important to differentiate this disorder from bronchial asthma.

Case Report

A 62-year-old woman was admitted to our hospital because of severe acute dyspnea, one month after she was referred to our hospital because of intractable bronchial asthma. Neither medical nor family history was contributory. She had a 22-year history of smoking (10 cigarettes/day). At the age of 59, she was admitted to another hospital with dyspnea, with no demonstration of saddle nose or flare/swelling of the ear auricles at that time. Based on elevated ST in an extensive area on electrocardiography and increases in serum and plasma biomarkers of cardiac injury, a tentative diagnosis of myocarditis was made in addition to bronchial asthma. Subsequently, asthma treatment with oral prednisolone was initiated. When prednisolone was decreased in dose or discontinued, her asthma condition was exacerbated until ventilator assistance was required. During this clinical

[1]Center for Pulmonary Diseases, National Hospital Organization Tokyo National Hospital, Japan, [2]Department of Allergy, National Hospital Organization Tokyo National Hospital, Japan and [3]Department of Clinical Research, National Hospital Organization Tokyo National Hospital, Japan

Received for publication February 28, 2012; Accepted for publication April 2, 2012

Correspondence to Dr. Nobuharu Ohshima, ohshima-in@tokyo-hosp.jp