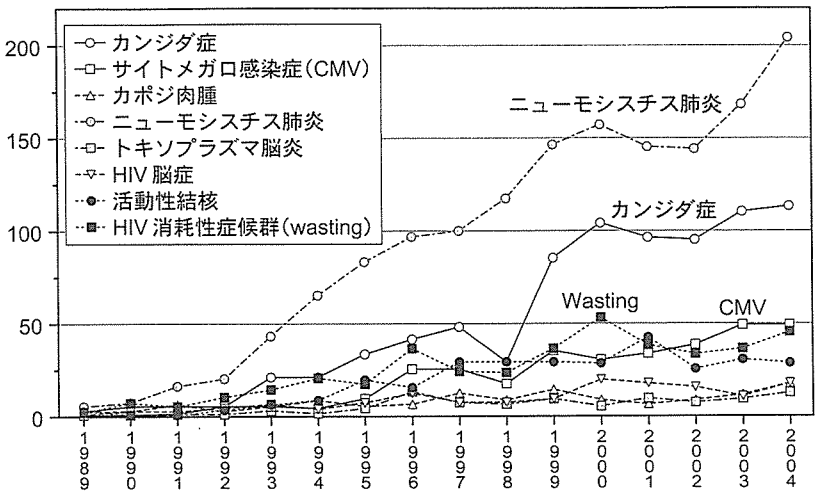

図1　日和見感染症の推移

報告されている日和見感染症は年々増加しており，特にニューモシスチス肺炎と（食道)カンジダ症で著しい。HIV 消耗性症候群は他疾患の除外診断によるので，報告時点で診断が確定していなかった症例が多く含まれている可能性がある。

（エイズ動向委員会報告）

を発症してからHIV感染がわかる，いわゆる“いきなりエイズ”が増加していることを反映したものと思われる。HIVと診断されて3カ月以内(同時を含む)の患者が70%以上を占めているが，2002年以降の特徴として，再びHIV感染症診断後1年以上経過した患者の頻度が増加してきている。一方HAART施行期間との関連では，HAARTが6カ月以上継続されている患者の割合は5％しかなく，ほとんどがHAART未施行，または中断中の患者である。診断後1年以上経過した患者でもHAART未施行か中断している例が多く，何らかの理由で定期受診をしていないか，HAARTが施行できない患者において，日和見感染症を発症する頻度が増加しつつあると考えられる。

日和見感染症の疾患では，図2のようにニューモシスチス肺炎が最も多く，サイトメガロウイルス感染症，結核の順となっている。この中で特にニューモシスチス肺炎は年々頻度が増してきており，“いきなりエイズ”の多くがニュー

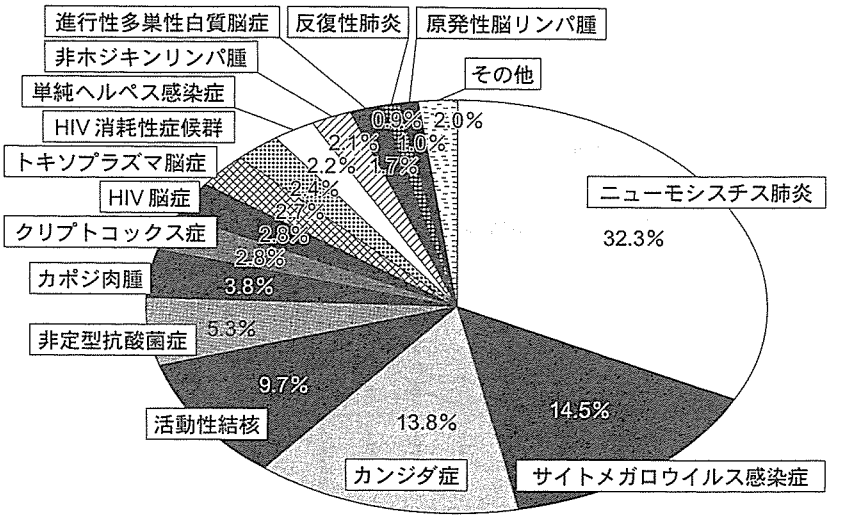

図2　エイズ指標疾患の頻度（1995～2004年）

ニューモシスチス肺炎，サイトメガロウイルス感染症，（食道）カンジダ症，活動性結核，非定型抗酸菌症の5疾患で，全体の75%を占めている。

（厚生労働省科研「HAART時代の日和見合併症」研究班）

モシスチス肺炎で発症している。また，カポジ肉腫や悪性リンパ腫，脳原発リンパ腫などの悪性腫瘍の頻度が，増加傾向にあるのも特徴的である。特にHIVに合併するリンパ腫は予後が悪く，研究班での調査でも死亡率は51%に上っており，今後注意が必要な疾患であるといえる。

まとめると，日本においては日和見感染症の報告数は増加してきており，ニューモシスチス肺炎や悪性リンパ腫など，いったん発症すると予後が悪い疾患が増加傾向にある。HIVの早期発見とHIVに合併する日和見感染症に対する診断技術の向上は，現在も重要な課題であるといえる。

（安岡　彰）

Q5 HIV感染で問題となる真菌症は？

▶A HIV感染症では，ウイルスの持続感染・増殖により細胞性免疫が破壊され，種々の日和見病原体に感染しやすくなる。特徴的な疾患に罹患するようになるとAIDS発症と診断されるが，この指標となる疾患が23あり，次の5つの真菌症が含まれる（表1）。

表1　AIDS発症診断の指標となる真菌症

ニューモシスチス肺炎
食道カンジダ症
クリプトコックス症（肺感染以外：髄膜炎や全身播種など）
ヒストプラズマ症
コクシジオイデス症

このほかにHIVによくみられる真菌症を示す（表2）。

表2　HIVによくみられる真菌症

口腔カンジダ症
膣カンジダ症
アスペルギルス症（肺や副鼻腔，脳）
マルネッフェイ型ペニシリウム症

ニューモシスチス肺炎は，以前原虫に分類されていた*Pneumocystis jiroveci*（以前は*carinii*）による重症肺炎で，AIDS発症の4～6割が本症の発症による。AIDS患者が本症を発症した場合，死亡率は10～20%と重篤な肺炎である。ニューモシスチスは真菌に属するものの，一般的な抗真菌薬は無効で，ST（sulfamethoxazole，trimethoprim：サルファメトキサゾール剤とトリメトプ

リム）合剤やペンタミジンによって治療を行う。治療開始に伴って生じる高度の炎症を抑えるために副腎皮質ステロイドホルモンを併用することで，以前と比べ大きく予後が改善した。

カンジダ症はもっとも頻度が高いHIV関連真菌症で，口腔内に粘膜の発赤を伴った白苔をきたすケースが一番よくみられる(口腔カンジダ症)。この病変が食道まで波及し，胸部の中心付近の痛み(胸骨裏面痛)や嚥下時痛をきたした場合が食道カンジダ症で，AIDS発症とみなされる。このほか，女性では腟カンジダ症もよく認められる。

カンジダ症の治療はアゾール系抗真菌薬の内服投与で，口腔のみの病変ではトローチやゲル製剤も有効である。治療は比較的容易で，治療開始後数日で粘膜所見の改善をみる。カンジダは皮膚や粘膜の常在菌であり，いったん治療してもくり返し発症しやすく，長期に渡る抗真菌薬の断続的使用から後述のように薬剤耐性が問題となることがある。

クリプトコックス症は，一般に，菌を含む粉塵を吸入することによって肺に初発病変を起こすことが多いが，HIV感染者では肺には明確な病変をきたすことはさほど多くなく，直接髄膜炎を起こしやすい。また，HIV感染者のクリプトコックス髄膜炎は，頭痛や嘔気といった髄膜刺激症状が軽度で，発熱と軽度の頭痛程度で髄膜炎としての所見が比較的乏しいまま，衰弱と意識障害が進むという経過をとることが多い。発見が遅れると播種性病変となっていて救命が困難であったり，一命をとりとめても著しい障害を残すことがあるなど，注意が必要な真菌症である。

ヒストプラズマ症は日本にはなく，輸入感染症として発症する。これまで外国人HIV感染者に発症したヒストプラズマ症が数例報告されている。

コクシジオイデス症はアメリカ大陸の風土病真菌症であり，日本ではHIV感染者での報告はまだない。

アスペルギルス症はAIDS指標疾患ではないが，高度免疫不全状態では肺や副鼻腔の感染が起こりやすい。高度免疫不全で死亡した例の解剖所見では，肺などにアスペルギルスの病変が認められることが少なくない。

マルネッフェイ型ペニシリウム症は，タイ北部からミャンマー，中国の雲南省にかけてみられる風土病的真菌症で，皮膚の結節病変や菌血症を起こす。この地域（特にタイ）から来日したHIV感染者では，考慮する必要がある。

HIVにみられる真菌症で問題となる点は，免疫不全が持続しているため治療への反応が悪く，長期治療が必要な点である。再燃・再発が多いため，免疫不全が改善しない限り，生涯の再発予防（2次予防）が必要である場合が多い。

また，口腔・食道カンジダ症などのようにくり返し治療が必要となる場合もあり，頻回に薬剤にさらされるため，薬剤に対する耐性を獲得することがある。アゾール（フルコナゾールなど）に対する *Candida albicans* の耐性化がよく知られており，この耐性はアゾール系薬すべてに交叉耐性を示す。治療は軽度耐性の場合は投与量増量で対応するが，高度耐性の場合はキャンディンやポリエンなど異なる系統の薬剤による治療が必要となる。

（安岡　彰）

Q36 口腔・食道カンジダ症の治療法は？

▶A　カンジダは口腔・食道の常在菌であるため，喀痰や口腔・食道拭い液などの微生物検査で陽性となっても，感染症を発症しているとはいえない。口腔カンジダ症・食道カンジダ症は，肉眼的あるいは内視鏡によって，口腔や食道に菌の増殖した淡黄白色の“白苔”の付着が確認され，粘膜の発赤，びらん，増殖性変化を伴った場合のみが治療対象となる(図1，2)。

口腔カンジダ症は軽度の免疫不全(高齢，糖尿病，副腎皮質ステロイドホルモンの吸入など）に加えて，口腔の保清が十分でない場合に発症する可能性がある。またHIV感染症では，免疫不全前駆症として重要であり，くり返す口腔

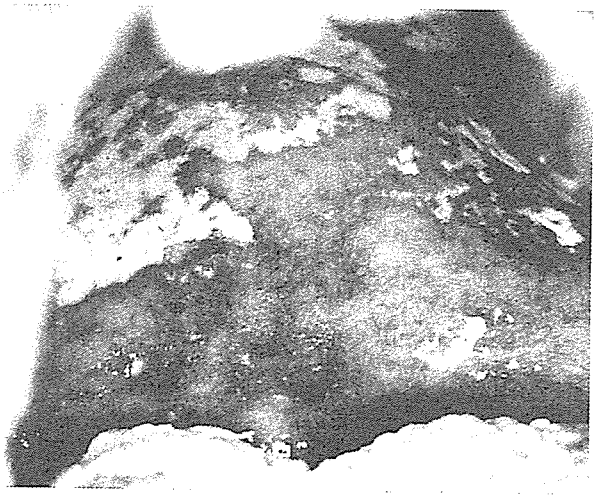

図1　口腔カンジダ症
軟口蓋から硬口蓋にかけて乳白色の白苔が付着し，周辺は発赤・びらんが認められる。舌にも厚い白苔がみられる。

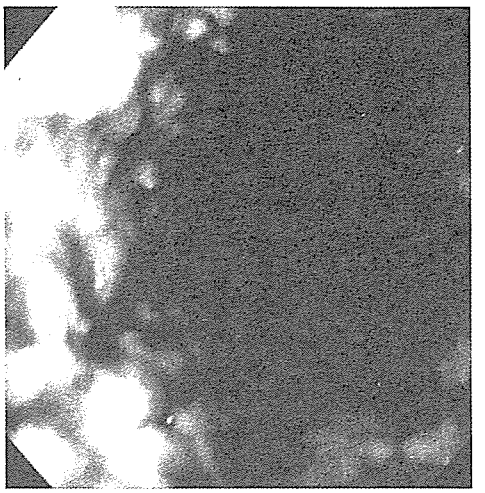

図2　食道カンジダ症
食道は全周性に厚い白苔に覆われ，正常粘膜は認められない。

カンジダ症をみた場合はHIV抗体検査をすすめるべきである。

食道カンジダ症は，ほとんどはHIV感染者でみられ，AIDS発症疾患の1つとされている。多くの患者では口腔カンジダ症もみられ，これに加えて前胸部の中心の痛み(胸骨裏面痛)が認められれば，臨床的には食道カンジダ症と診断される。しかし，HIV感染者に上部消化管内視鏡を行ってみると，口腔にはカンジダの病変がなくても高度の食道病変がみられる場合もあるため，胸骨裏面痛や嚥下痛，嚥下困難，原因不明のやせがみられる場合には積極的な内視鏡検査がすすめられる。

口腔カンジダ症の治療には，アゾール系抗真菌薬の経口的全身投与と，トローチやシロップ剤による局所投与がある。前者はすみやかに効果が期待できるが，肝障害などの副作用を考慮する必要があり，後者は全身の副作用はほとんどみられないが，軽快までにやや時間を要し，また1日4～6回の服用が必要と，やや煩雑である。投与法および投与量は次の通りである。

・フルコナゾール（ジフルカン®）
　50～100 mg　1日1回　7～14日
・イトラコナゾール（イトリゾール®）
　50～200 mg　1日1回　7～14日
・クロトリマゾールトローチ（エンペシドトローチ®）
　1回1錠1日5回口腔内で溶解
・ミコナゾールゲル（フロリードゲル®）
　1回10～20 mLを口腔内に含んだ後嚥下1日4回

食道カンジダ症は口腔カンジダ症で用いられる局所治療薬は無効で，アゾール系抗真菌薬の経口投与が行われる。経口摂取困難な場合は，治療開始数日は点滴投与も行われる。第一選択はフルコナゾール（ジフルカン® またはプロジフ®）100～200 mg/日を1日1回，1～3週間投与する。

いずれのカンジダ症も，口腔・食道にびらんや潰瘍を形成する疾患との鑑別が重要となる。口腔では単純ヘルペスや特発性の口腔アフタ，梅毒・クラジミアの咽頭病変など，食道ではサイトメガロウイルス食道潰瘍や単純ヘルペス，HIVウイルスによる食道潰瘍などが挙げられる。

HIVにみられる口腔・食道カンジダ症は反復性であるが，比較的容易に治療が可能であり，通常，発症予防投与は行わない。しかし，治療終了後すぐに再発する場合や難治例では，フルコナゾール100～200 mg/日の持続的な予防投与が考慮される。

HIV感染者のカンジダ症では，アゾール系抗真菌薬を長期投与することになるため，アゾール系抗真菌薬に対する耐性を獲得することがあり，注意を要する（Q 5・Q 15参照）。

（安岡　彰）

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yasuhisa Abe, Daisuke Matsubara, Hiroyuki Gatanaga, Shinichi Oka, Satoshi Kimura, Yuki Sasao, Kiyoshi Saitoh, Takeshi Fujii, Yuko Sato, Tetsutaro Sata and Harutaka Katano	Distinct expression of Kaposi's sarcoma-associated herpesvirus-encoded proteins in Kaposi's sarcoma and multicentric Castleman's disease	Pathology International	56	617-624	2006
Md. Zahidunnabi Dewan, Hiroshi Terunuma, Masakazu Toi, Yuetsu Tanaka, Harutaka Katano, Xuewen Deng, Hiroyuki Abe, Tadashi Nakasone, Naoki Mori, Tetsutaro Sata and Naoki Yamamoto	Potential role of natural killer cells in controlling growth and infiltration fo AIDS-associated primary effushion lymphoma cells	Cancer Sci	97	1381-1387	2006
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Hiroshi Minoda, Norio Usui, Tetsutaro Sata, Harutaka Katano, Hiromi Serizawa and Shinya Okada	Human Herpesvirus-8 in Kaposi's Sarcoma of the Conjunctiva in a Patient with AIDS	Jpn J Ophthalmol	50	7-11	2006
Y Yanagisawa, Y Sato, Y Asahi-Ozaki, E Ito, R Honma, J Imai, T Kanno, M Kano, H Akiyama, T Sata, F Shinkai-Ouchi, Y Yamakawa, S Watanabe and H Katano	Effusion and solid lymphomas have distinctive gene and protein expression profiles in an animal model of primary effusion lymphoma	J Pathol	209	464-473	2006

Pathology International 2006; **56**: 617–624 doi:10.1111/j.1440-1827.2006.02017.x

Case Report

Distinct expression of Kaposi's sarcoma-associated herpesvirus-encoded proteins in Kaposi's sarcoma and multicentric Castleman's disease

Yasuhisa Abe,[1] Daisuke Matsubara,[2] Hiroyuki Gatanaga,[1] Shinichi Oka,[1] Satoshi Kimura,[1] Yuki Sasao,[2] Kiyoshi Saitoh,[2] Takeshi Fujii,[2] Yuko Sato,[3] Tetsutaro Sata[3] and Harutaka Katano[3]

[1]*AIDS Clinical Center and* [2]*Department of Pathology, International Medical Center of Japan and* [3]*Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan*

The expression of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8)-encoded proteins is herein demonstrated in Kaposi's sarcoma (KS) and multicentric Castleman's disease (MCD) in a single lymph node derived from a patient with acquired immunodeficiency syndrome. Immunohistochemistry revealed that both lytic and latent KSHV proteins were expressed in cells of the MCD lesion. KSHV-encoded viral interleukin-6 was also detected in follicular dendritic cells of the germinal center. Cytoplasmic localization of open reading frame 59 protein and latency-associated nuclear antigen suggested KSHV activation in the MCD lesion. Moreover, a high copy number of KSHV was detected in the blood. Clinically, pegylated-liposomal doxorubicin induced regression of not only KS, but also lymphadenopathy of the MCD lesion with a decrease in KSHV load and human interleukin-6 in the blood. To the best of the authors' knowledge this is the first case demonstrating differential expression of virus proteins in two KSHV-associated diseases, KS and MCD, in the same section. The case confirms lytic KSHV infection in MCD, and suggests that clinical symptoms of MCD might be closely linked with KSHV activation.

Key words: Kaposi's sarcoma-associated herpesvirus, Kaposi's sarcoma, multicentric Castleman's disease, open reading frame 59, viral interleukin-6

Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) infection is associated with the pathogenesis of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL; body cavity-based lymphoma) and some cases of multicentric Castleman's disease (MCD).[1–5] KSHV, a large DNA virus, encodes more than 80 viral genes/proteins in its genome. Similar to other herpes viruses, KSHV infection can be characterized as lytic or latent.[4] During lytic infection, KSHV is activated, producing numerous lytic proteins, and virions are packaged and released from the cells. In this process, DNA synthesis and expression of virion structural protein genes are required. Latent infection is characterized by persistence of the viral genomes as a covalently closed circular episome with limited viral gene expression.[4,6]

The differences between these two types of infection are strongly associated with their clinical courses. KSHV-associated malignancies such as KS and PEL are associated with latent KSHV infection. KS is one of the major opportunistic tumors in homosexual acquired immunodeficiency syndrome (AIDS) patients.[7] Previous immunohistochemistry showed that almost all spindle cells in KS expressed KSHV-encoded latency-associated nuclear antigen (LANA), whereas expression of lytic proteins such as open reading frame (ORF) 59, ORF50, and viral interleukin-6 (vIL-6) were rare in the KS cells, implying that KS cells are infected with KSHV in the latent phase.[1–3] Although a small number of PEL cells express several lytic KSHV proteins, almost all usually express LANA in the nucleus, suggesting that latent KSHV infection is predominant among PEL cells. Thus, latent KSHV infection plays an important role in the pathogenesis of KSHV-associated malignancies.

In contrast, the pathogenesis of lytic KSHV infection is relatively unclear. MCD is a rare systemic lymphoid hyperplasia characterized by constitutional symptoms such as generalized lymphadenopathy, hepatosplenomegaly and polyclonal hyperimmunoglobulinemia and a high level of serum interleukin-6 (IL-6).[8–10] Follicular hyperplasia with proliferation of plasma cells and hyaline vascular alterations in the lymph node are histological hallmarks of MCD. KSHV is frequently detected in MCD tissues obtained from patients with human immunodeficiency virus (HIV) infection.[5,11–13] Lytic gene products encoded by KSHV are frequently expressed by B cells in the mantle zone of MCD lesions. This

Correspondence: Harutaka Katano, DDS, PhD, Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan. Email: katano@nih.go.jp

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observation is compatible with the finding that KSHV-positive PEL cells express CD138, but not Bcl6,[14] suggesting that KSHV infects post-germinal center B cells in the lymph node. The KSHV-positive cells in MCD lesion express various lytic proteins such as ORF50, ORF59, ORF65 and K8 proteins, whereas those expressions are very rare in KS lesions. These data suggest the predominance of lytic infection and a different pathogenesis from KSHV-associated malignancies such as KS and PEL.[3]

KS has frequently been reported among AIDS patients with MCD.[8] Here, we present a case of MCD complicated with KS in a single lymph node. This case clearly showed differential expression of viral proteins in two KSHV-associated diseases, providing the evidence that KSHV is highly activated in patients with MCD and strongly suggesting that MCD is related to acute KSHV infection.

CLINICAL SUMMARY

A 35-year-old homosexual Japanese man was admitted to AIDS Clinical Center with a 5 month history of high fever, lymphadenopathy, bicytopenia (anemia and thrombocytonemia), splenomegaly, purple macula and hoarseness. His body temperature was 40°C. Many lymph nodes, each >2 cm in diameter, were palpable in cervical, axillary and inguinal lesions. Macula was observed on the face, limbs, soma and also in the oral cavity. Laboratory analysis indicated anemia (hemoglobin 7.5 g/dL), severe thrombocytopenia (platelets 3.8×10^4/mL), elevated transaminase (aspartate aminotransferase 80 IU/L, alanine aminotransferase 87 IU/L), and CRP 6.17 mg/dL. Chronic Epstein–Barr virus (EBV) infection was detected by serology and polymerase chain reaction (PCR). Serological and PCR assays demonstrated that the patient was HIV-1 positive. Biopsy revealed involvement of KS cells in the axillary lymph node. Because of severe thrombocytopenia and anemia with development of KS and lymphadenopathy, blood transfusions (red blood cells and platelets) were performed several times. Two weeks after admission, the patient's hoarseness worsened and he complained of dyspnea. His airway was shown to be almost completely obstructed by larynx edema due to KS lesions. A tracheotomy was performed to maintain his airway and pegylated-liposomal doxorubicin (Doxil 20 mg/mm^2; Alza, Mountain View, CA, USA) was initiated at 2 week intervals. One week after the introduction of this treatment, his larynx edema and general lymphadenopathy were resolved. No evidence of recurrence was observed during the following 6 months.

MATERIALS AND METHODS

All samples including biopsy and blood were taken from the patient under informed consent. Serial sections were prepared and stained with HE for light microscopy, or subjected to immunohistochemistry. Immunohistochemical staining was visualized using the biotin–streptavidin–peroxidase method with 3-3'diaminobenzidine as the chromogen, as previously described.[3] Anti-KSHV-LANA, ORF59, K8, and vIL-6 rabbit polyclonal antibodies established by our group in previous studies[3,15,16] were used as primary antibodies.

KSHV-positive PEL cell lines, TY-1 and BCBL-1, were cultured in RPMI 1640 containing 10% fetal bovine serum in 5% CO_2 at 37°C.[17,18] Twenty ng/mL of tetradecanoylphorbol acetate (TPA) was added to the culture medium. TY-1 and BCBL-1 were spotted onto glass slides and fixed with cold acetone for 10 min. Diluted anti-ORF59 rabbit polyclonal antibody[16] or patient serum was then reacted for 60 min in a moisture chamber. The slides were washed and fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG or antihuman Igs (Biosource International, Camarillo, CA, USA) was applied as the second layer for 30 min at 37°C. After counterstaining with propidium iodide (PI, 50 μg/mL), the slides were mounted with buffered glycerin. Imaging was performed using a confocal microscope equipped with an argon-krypton laser (LSM-MicroSystem, Carl-Zeiss, Jena, Germany). PI and FITC were stimulated at 488 nm and the emission patterns of the two fluorescences were collected separately and overlaid using a computer to create two-color images.

Serum antibodies to KSHV were detected with enzyme-linked immunosorbent assay and immunofluorescence assay as previously described.[19] Virus copy number in the blood was detected with real-time PCR as previously described.[20]

PATHOLOGICAL FINDINGS

Detection of both KS and MCD in a single lymph node

Histologically, a biopsy sample of the axillary lymph node demonstrated large lymphoid follicles and KS spindle cell proliferation (Fig. 1a). KS spindle cells proliferated and formed interweaving fascicles with vascular slits mainly in the sinus, expanding to the interfollicular space of the lymph node and infiltrating the surrounding fat tissue. Vascular slits formed by KS spindle cells contained erythrocytes configured linearly (Fig. 1c). Extravasated erythrocytes were abundant between KS spindle cells, and they were sometimes phagocyted by macrophages. Some hyaline globules and mitotic figures were identified in the KS lesion. The biopsy sample also showed partly involuted germinal centers surrounded by small lymphocytes and a hyaline vascular change-like onion skin appearance (Fig. 1a). Proliferation of small vessels in the follicles and perifollicular area was observed, and plasma cell proliferation was noted in the interfollicular space. These observations fitted the histological criteria of MCD.[8,9] These two lesions, KS and MCD, were present side by side, but not

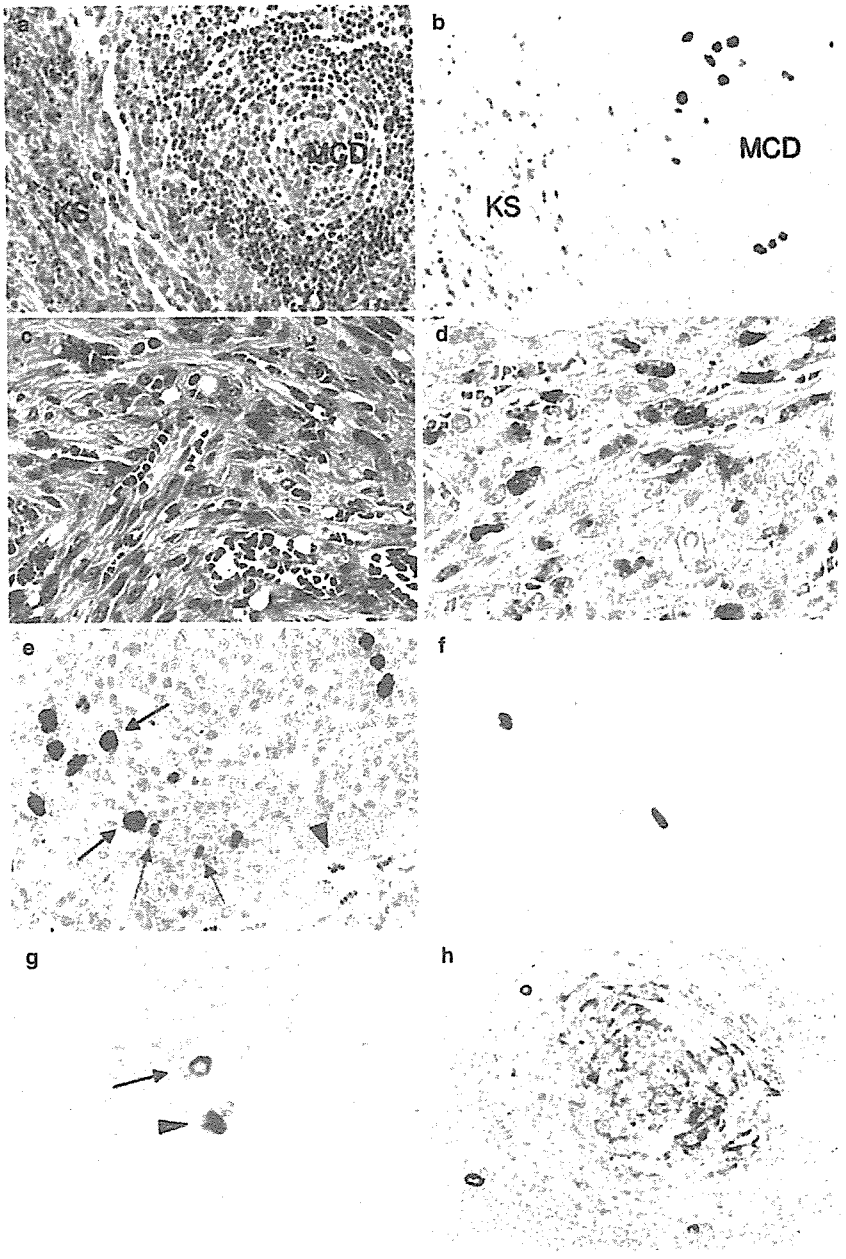

Figure 1 Histology of the axillary lymph node. Original magnifications: ×100 (**a,b,h**) and ×400 (**c–g**). (**a**) Low-power view of the lymph node. HE stain. KS, Kaposi's sarcoma; MCD, multicentric Castleman's disease. (**b**) Immunohistochemistry for latency-associated nuclear antigen (LANA). Both the KS and MCD lesions contain LANA-positive cells. (**c**) HE stain of KS lesion. KS spindle cells proliferate with vascular slits. (**d**) LANA is detected in the KS cells as dot-like staining in the nucleus. (**e**) LANA in the MCD lesion. Some cells express LANA as dot-like staining in the nucleus (red arrows), whereas a large number of the LANA-positive cells demonstrate a diffuse staining pattern in the cytoplasm and nucleus (arrows). Arrowheads indicate staining of LANA in the KS lesion. (**f**) Open reading frame (ORF)59 expression in the KS lesion. (**g**) ORF59 expression in the MCD lesion. ORF59 is expressed in the cytoplasm (arrow) or nucleus (arrowhead). (**h**) Viral interleukin-6 expression in the MCD lesion.

mixed. KS spindle cells were not found in the lymphoid follicles with MCD. However, proliferation of plasma cells in the interfolliclar space was found close to KS lesions. Thus, the lymph node was determined as containing both KS and MCD lesions.

Differential expression of viral proteins in KS and MCD

Thus far, no case of coexisting KS and MCD in the same lymph node of an AIDS patient has been reported. Differential expression of viral proteins have been demonstrated in KS and MCD samples, but these studies were performed using different sections from different patients.[1–3] To confirm the expression of viral proteins in KS and MCD lesions, we performed immunohistochemistry of the section in which KS and MCD were shown to coexist. Immunohistochemistry revealed KSHV-encoded vIL-6, ORF 50, ORF59, K8, and LANA expression in mantle zone cells in the MCD lesion (Fig. 1 and data not shown). Interestingly, KSHV-encoded vIL-6 was also detected in follicular dendritic cells (FDC) in

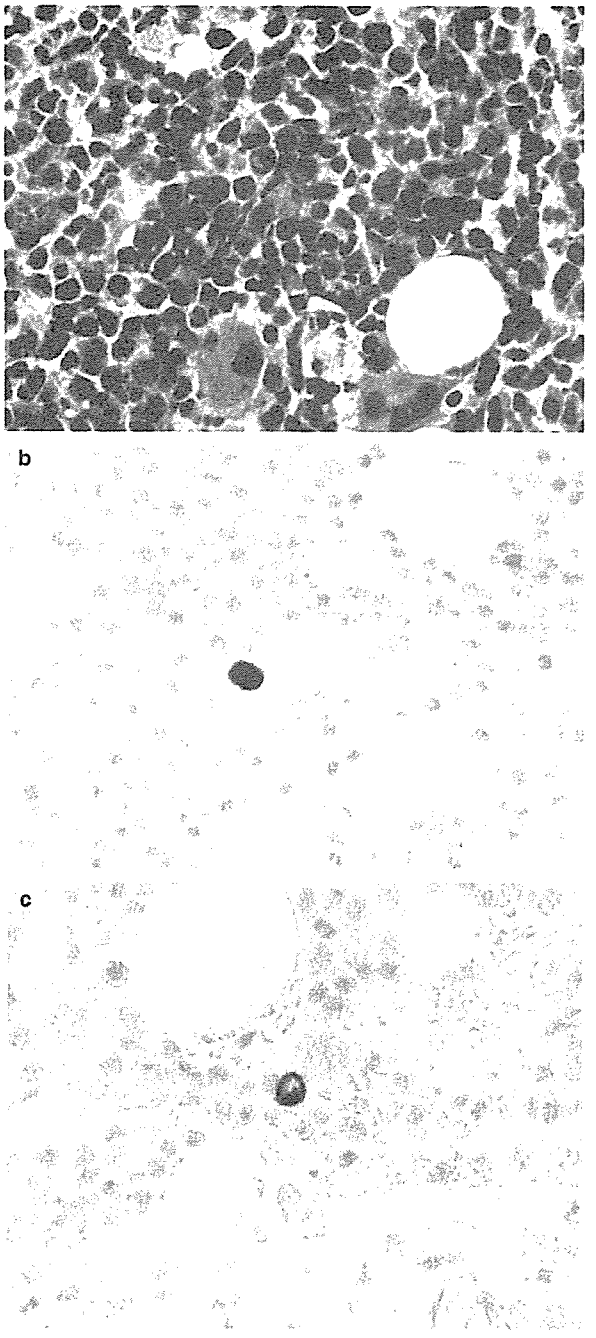

Figure 2 Histology of the bone marrow. Original magnification: ×400. (**a**) HE stain. Plasma cell proliferation is shown in the center of the panel. (**b**) Immunohistochemistry for latency-associated nuclear antigen (LANA). A small number of bone marrow cells express LANA. (**c**) A few cells express open reading frame (ORF)59 protein in the cytoplasm.

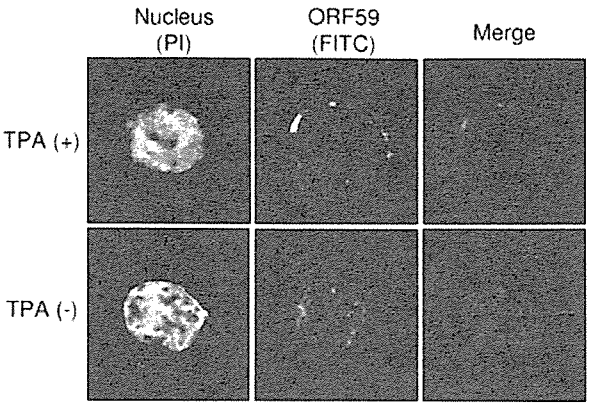

Figure 3 Expression of the open reading frame (ORF)59 protein in tetradecanoylphorbol acetate (TPA)-stimulated and unstimulated Kaposi's sarcoma-associated herpesvirus (KSHV)-infected primary effusion lymphoma (PEL) cell lines. A KSHV-positive cell line, TY-1, was stimulated with TPA then smeared on a slide glass. ORF59 protein is stained with anti-ORF59 rabbit polyclonal antibody as the primary antibody. Fluorescein isothiocyanate (FITC)-labeled antirabbit Ig antibody was used as the secondary antibody (green). The nucleus was counterstained with propidium iodide (PI; red). A small number of unstimulated TY-1 cells expressed ORF59 protein in the nucleus (lower panels). Although the nuclear localization of ORF59 protein was predominant in TPA-stimulated TY-1, some of the stimulated TY-1 cells expressed ORF59 protein in the cytoplasm (upper panels). BCBL-1 demonstrated similar results (data not shown). Original magnification: ×400.

the germinal center of MCD (Fig. 1h). Because the FDC were negative for LANA (Fig. 1b,e), it is suggested that a large amount of vIL-6 was trapped by the FDC in the form of immune complex. In contrast, the KS cells expressed LANA with rare expression of lytic proteins (Fig. 1b,e–g). Immunohistochemistry also revealed different subcellular localization of LANA between KS and MCD (Fig. 1e). LANA expression was localized in the cytoplasm and nucleus in MCD (Fig. 1e), but in the nucleus only as a dot-like staining pattern in the KS cells (Fig. 1e). Subcellular localization of ORF59 protein was also different between KS and MCD. In MCD, expression was observed in the cytoplasm or nucleus of mantle zone B cells (Fig. 1g); however, expression was confined to the nucleus in only a few KS cells, with no expression in the cytoplasm (Fig. 1f). These data clearly demonstrate the distinctive expression of viral proteins in KS and MCD lesions.

KSHV-infected cells in the bone marrow

Histology of a bone marrow biopsy showed hypercellular features with plasma cell proliferation (Fig. 2a), but there was no histological evidence of MCD or lymphoma in the bone marrow. Immunohistochemistry showed a small number of LANA-positive cells (Fig. 2b). Interestingly, ORF59-positive

signals were also found in the cytoplasm of the bone marrow cells (Fig. 2c). In this case, severe anemia and thrombocytopenia were also seen, whereas histology of the bone marrow showed trilineage hematopoiesis. No evidence of hemophagocytosis was found.

Cytoplasmic localization of ORF59 in KSHV-infected PEL cell lines

ORF59 protein is known as a DNA-processing factor of KSHV.[21] During replication of KSHV-DNA in infected cells, ORF59 protein processes DNA synthesis with KSHV-encoded DNA polymerase (ORF9). Thus, ORF59 protein is expressed in the lytic phase of KSHV infection and is required for DNA replication of KSHV. Because the *ORF59* gene has a nuclear localization signal in its sequence, transfection of the *ORF59* gene to mammalian cells results in nuclear localization of the ORF59 protein.[22] However, immunohistochemistry demonstrated that a large number of the ORF59-positive cells in the mantle zone of the MCD lesion expressed ORF59 protein in the cytoplasm (Fig. 1f). Moreover, ORF59-positive cells in the bone marrow also demonstrated cytoplasmic localization of the ORF59 protein (Fig. 2c).

To assess the different subcellular localizations of ORF59 protein in KSHV infection, we investigated expression of the ORF59 protein in KSHV-infected PEL cell lines, TY-1 and BCBL-1, with or without phorbol ester stimulation. An immunofluorescence assay demonstrated that TPA induced expression of ORF59 protein in 30–50% of the cells. The localization of ORF59 protein was predominantly in the nucleus and occasionally in the cytoplasm (Fig. 3).[3,23] A small portion (<5%) of unstimulated PEL cells also expressed the ORF59 protein but they were located only in the nucleus. Thus, these data suggest that cytoplasmic localization of the ORF59 protein is seen only in lytic KSHV infection.

KSHV load in the blood and the link with clinical symptoms

Based on the histology and immunohistological data, KSHV was suggested as being highly activated in this patient. To confirm the activity of KSHV, KSHV load in the blood was monitored. Real-time PCR showed a high titer of KSHV-DNA in the blood (8.0×10^3 copies/mL) on admission (Fig. 4a). However, after treatment with pegylated-liposomal doxorubicin, the KS lesion rapidly regressed and the KSHV load dramatically decreased to 2×10^2 copies/mL (Fig. 4a). Serum hIL-6 also decreased from 19.6 pg/mL to 5.2 pg/mL. Retrospective analysis using stored serum samples showed that this patient did not have KSHV IgM antibody, but rather possessed KSHV IgG antibody (Fig. 4b). Titers of KSHV antibody gradually reduced during admission (Fig. 4a). These data suggest that the KSHV load in the blood was tightly linked with the clinical symptoms of this patient.

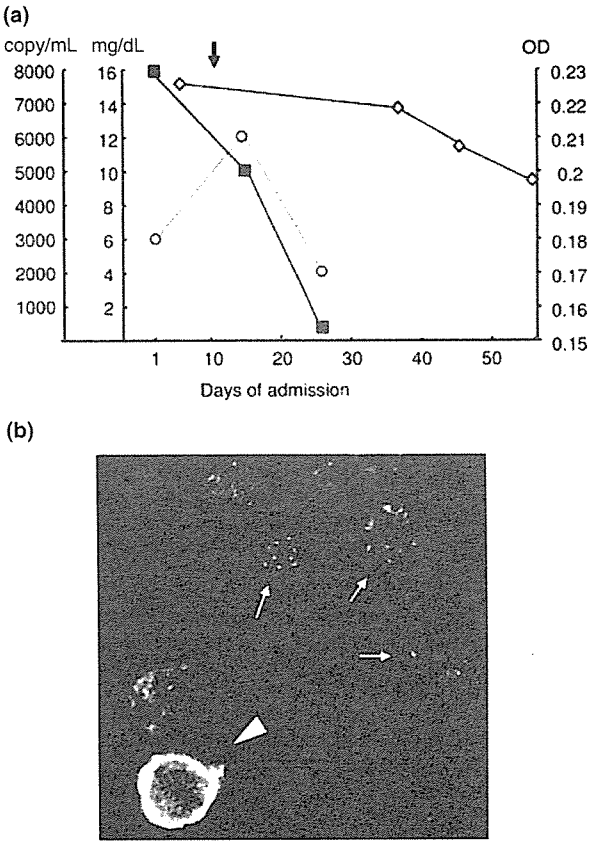

Figure 4 Kaposi's sarcoma-associated herpesvirus (KSHV) infection and the clinical course of (**a**) KSHV load (■; copy/mL), KSHV antibody (◇; optical density (OD); cut-off = 0.1) in the blood, and CRP (○; mg/dL). Liposomal doxorubicin was introduced on day 15 (arrow). (**b**) Results of immunofluorescense assay. Tetradecanoylphorbol acetate-stimulated TY-1 cells were stained with the patient's serum as the primary antibody and fluorescein isothiocyanate-conjugated antihuman Igs goat antibody was used as the secondary antibody. The serum contained not only antibodies to latency-associated nuclear antigen (arrows), but also antibodies to lytic KSHV protein (arrowhead). Original magnification: ×400.

DISCUSSION

In the present study we demonstrated MCD with a KS lesion in an HIV-positive patient. To the best of our knowledge, this is the first study to immunohistochemically demonstrate differential expression of virus proteins in two KSHV-associated diseases, KS and MCD, in the same section. In addition to

differential expression of lytic KSHV proteins in KS and MCD, several novel features of MCD were seen: (i) accumulation of vIL-6 in FDC; (ii) cytoplasmic localization of ORF59 protein specific to activated KSHV-infected cells; (iii) KSHV-infected cells in the bone marrow; and (iv) a high copy number of KSHV in the blood and a link with the clinical symptoms. These observations suggest that MCD is related to the lytic phase of KSHV infection, whereas KS is related to chronic infection. These data are likely to be useful in the treatment of both KS and MCD, suggesting, as it does, that reducing KSHV load might be important for treatment of these diseases with lytic KSHV infection.

MCD is a very rare disease among immunocompetent individuals, and the incidence among HIV-infected patients is much higher than among the general population.[8] In HIV-infected patients, MCD is generally observed in conjunction with KS.[8] KSHV has been detected frequently in HIV-infected patients with MCD, although KSHV infection is rare among immunocompetent patients with MCD.[13] KSHV infection in MCD was previously shown to differ from that in KS not only with regard to the infected cell type but also viral gene expression.[1–3] The findings of lytic and latent proteins in the present study were compatible with the results reported previously.[1–3]

KSHV-infected cells express various kinds of lytic proteins encoded by KSHV, such as ORF50, ORF59 and K8, in mantle zone B cells in MCD lesions. In contrast, KS cells express only LANA, predominantly in the nucleus, and expression of lytic proteins is rare. In the present study we demonstrated subcellular localization of viral proteins in MCD (B cells) and KS (KS cells). Among the viral proteins, subcellular localization of ORF59 protein is important for recognition of the status of virus infection. Our *in vitro* study (Fig. 3) and previous reports showed that the cytoplasmic expression of ORF59 protein was induced only in lytic infection.[3,23] In the present case, the cytoplasmic expression of ORF59 protein was observed only in the MCD lesion, not the KS lesion, suggesting replication of the virus in the former lesion only. Like ORF59, cytoplasmic expression of LANA in B cells of MCD lesions also suggests the lytic replication of the infected cells. Thus, the present case clearly demonstrated differential expression of viral proteins in two KSHV-associated diseases, suggesting different roles of KSHV in the pathogenesis of KS and MCD.

vIL-6 is thought to play an important role in the pathogenesis of MCD[12,24] and has been shown to act as an autocrine or paracrine factor of lymphoproliferative conditions.[25] vIL-6 has approximately 50% similarity with the hIL-6 gene at the amino acid level,[26] and was previously shown to induce the production of hIL-6 in cells harvested from the lymph node of a patient with MCD.[27] In the present case, cells in the mantle zone of MCD expressed vIL-6 in the cytoplasm. Moreover, vIL-6 was detected in FDC in the germinal center. FDC trap various antigens in the form of immune complex and present them to B cells,[28] and many viral antigens such as HIV p24 have been detected in FDC in the lymph nodes.[29] This is the first case demonstrating vIL-6 in the FDC of MCD. It was clear that the FDC were not infected with KSHV, because they were negative for LANA. Thus, the staining of vIL-6 in the FDC suggests accumulation of vIL-6-immunocomplex in FDC because of a high level of vIL-6 in the serum or lymph fluid. In this case, hIL-6 was also elevated before pegylated-liposomal doxolubicin administration and an increased number of plasma cells was also detected in the bone marrow cells. Surprisingly, KSHV-infected cells were also detected in the bone marrow. KSHV-positive cells have been reported in the bone marrow of patients with MCD, suggesting that plasmablastic cells or bone marrow stromal cells were positive for KSHV.[30,31] In the present case, KSHV-infected cells in the bone marrow also expressed ORF59 protein in cytoplasm (Fig. 2c). Considering these data, it was suggested that KSHV infection and vIL-6 expression played a role in the pathogenesis of this aggressive case.

MCD has a poor prognosis with a rapidly fatal clinical course.[8] Although there is no standard treatment for MCD, symptomatic cases are often treated with chemotherapy,[32,33] corticosteroids,[33–35] interferon-α,[36] monoclonal antibody to hIL-6[37–39] or anti CD20 monoclonal antibodies.[40,41] In the present study serum KSHV concentrations were shown to be linked with the clinical symptoms of MCD, and a recent study revealed that antiviral agents against KSHV were effective in three patients with KSHV-associated MCD.[42] In the present case, pegylated-liposomal doxorubicin induced regression of not only KS, but also lymphadenopathy of the MCD lesion. Currently pegylated-liposomal doxorubicin is the treatment of choice for KS,[43] but it has rarely been reported as a treatment for MCD. Administration of pegylated-liposomal doxorubicin has been shown to reduce KSHV load,[44,45] and in the present case administration caused a decrease in KSHV copy number with a reduction in the KS lesion, resulting in a favorable outcome. Thus, pegylated-liposomal doxorubicin had an effect not only on KS in the present case, but also on MCD by reducing the viral load in the blood, suggesting a possible therapy for complications associated with KS and MCD. Antiviral drugs might be effective in reducing the viral load in the blood with MCD, but not KS, and therefore a combination of pegylated-liposomal doxorubicin and antiviral drugs is considered a treatment option for cases of KS with MCD. However, further case studies will be needed to assess the effectiveness of this therapy.

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Potential role of natural killer cells in controlling growth and infiltration of AIDS-associated primary effusion lymphoma cells

Md. Zahidunnabi Dewan,[1,2] Hiroshi Terunuma,[3,4] Masakazu Toi,[5] Yuetsu Tanaka,[6] Harutaka Katano,[7] Xuewen Deng,[3] Hiroyuki Abe,[4] Tadashi Nakasone,[2] Naoki Mori,[8] Tetsutaro Sata[7] and Naoki Yamamoto[1,2,9]

[1]Department of Molecular Virology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519; [2]AIDS Research Center, National institute of Infectious Disease, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640; [3]Biotherapy Institute of Japan, 2-4-8 Edagawa, Koutou-ku, Tokyo 135-0051; [4]Kudan Clinic, 1-9-5 Kudankita, Chiyoda-ku, Tokyo 102-0073; [5]Division of Clinical Trials and Research, Breast Cancer Research and Treatment Program, Tokyo Metropolitan Komagome Hospital, Tokyo Medical Center for Cancer and Infectious Disease, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677; [6]Department of Immunology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215; [7]Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjyuku-ku, Tokyo 162-8640; [8]Division of Molecular Virology and Oncology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

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Natural killer (NK) cells are an important component of the innate immune response against microbial infections and tumors. Direct involvement of NK cells in tumor growth and infiltration has not yet been demonstrated clearly. Primary effusion lymphoma (PEL) cells were able to produce tumors and ascites very efficiently with infiltration of cells in various organs of T-, B- and NK-cell knock-out NOD/SCID/γc^{null} (NOG) mice within 3 weeks. In contrast, PEL cells formed small tumors at inoculated sites in T- and B-cell knock-out NOD/SCID mice with NK-cells while completely failing to infiltrate into various organs. Immunosupression of NOD/SCID by treatment with an antimurine TM-β1 antibody, which transiently abrogates NK cell activity *in vivo*, resulted in enhanced tumorigenicity and organ infiltration in comparison with non-treated NOD/SCID mice. Activated human NK cells inhibited tumor growth and infiltration in NOG mice. Our results suggest that NK cells play an important role in growth and infiltration of PEL cells, and activated NK cells could be a promising immunotherapeutic tool against tumor or virus-infected cells either alone or in combination with conventional therapy. The rapid and efficient engraftment of PEL cells in NOG mice also suggests that this new animal model could provide a unique opportunity to understand and investigate the mechanism of pathogenesis and malignant cell growth. (*Cancer Sci* 2006; 97: 1381–1387)

Primary effusion lymphoma (PEL) was originally identified in AIDS-associated immunodeficient patients and has been recognized by the World Health Organization as a distinct AIDS-related form of B-cell lymphoproliferative disorder.[1–3] PEL is a non-Hodgkin's type lymphoma derived from postgerminal center B cells.[4] The tumor clone is characteristically infected by the Kaposi's sarcoma-associated herpesvirus, formerly called human herpesvirus type 8 (HHV-8),[5] and most cases are coinfected with Epstein–Barr virus.[6,7] PEL shows a peculiar presentation involving lymphomatous effusions of serous cavities and only occasionally presents with a definable mass.[5]

Immunodeficient mouse models of human malignancy have contributed significantly to understanding the pathogenesis of diseases as well as therapeutic purpose. The congenitally athymic and hairless nude mouse lacking functional T cells has been utilized as a host for human xenotransplantations for 30 years.[8] Thereafter, severe combined immunodeficiency (SCID) mice were found to have a genetic defect preventing functional development of T and B lymphocytes,[9,10] and can be engrafted successfully with a variety of normal hematopoietic and neoplastic cells.[11,12] In comparison with conventional SCID, the NOD-SCID strain appears to be more promising as a tool for xenotransplantion of human tumors. However, the NOD-SCID mouse strain retains natural killer (NK) cell activity, macrophage function, complement activity and functional dendritic cells.[13] NK cells might play an important role in the rejection of implanted tissues or cells in SCID mice.[14–17] Although several models using mainly conventional nude and SCID mice are available, there are some major drawbacks: the requirement of long time periods, repeated transplantation, total body irradiation of mice, hormone supplements, etoposide pretreatment and anti-NK monoclonal antibodies required for tumor formation. These problems appear to hinder wider use of these animal models. Due to the low engraftment efficiency of hematopoietic and tumor cells transplanted in SCID mice, T, B and NK knock-out NOD/SCID/γc^{null} (NOG) mice were used in the present study to investigate the role of NK in tumor growth and metastasis.[13]

Natural killer cells are a type of lymphocyte that comprises up to 15% of peripheral blood lymphocytes and mediates innate immunity against pathogens and tumors.[17] In addition, NK cells are an important source of cytokines that regulate hematopoiesis and link the innate to the adaptive immune response through a bidirectional cross-talk with dendritic cells.[18,19] NK cells were originally discovered because of their ability to kill tumor and virally infected cells *in vitro*. NK-cell activity against these *in vitro* targets is spontaneous; it is readily apparent in individuals who have not been previously exposed to the target cell antigens. A clear involvement of NK cells in antitumor immunity *in vivo*, and the involvement of major histocompatibility complex (MHC) class I in NK-cell recognition, was shown in 1986 by Karre and colleagues.[20] They showed that the RMA T-cell lymphoma, derived from the Rauscher virus-induced murine cell line RBL-5, grew progressively in syngenic mice, but that an MHC class I-negative variant, RMA-S, was rejected by host NK cells. In many different situations, NK cells were shown to kill certain tumor cell lines *in vitro*, despite significant levels of MHC class I on their cell surface.[21,22] This implied that killing of MHC class I^+ tumor cells was mediated by activating receptors that were either not impaired by the inhibitory NK receptors for MHC class I or provided sufficient stimulation to overcome the negative regulation.

One cohort study showed that individuals with low natural cytotoxic activity of peripheral blood lymphocytes are at a

[9]To whom correspondence should be addressed. E-mail: yamamoto.mmb@tmd.ac.jp

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significantly higher risk of cancer, compared with those of median or high activity.[23] It has been reported recently that NK cells isolated from HIV-infected individuals are impaired in their ability to kill the virus-infected autologous cells, as well as tumor cell lines.[24–27] Previous studies also reported that NK-cell activity controls PEL and Kaposi's sarcoma (KS) development associated with HHV-8 infection.[28,29] The ability of the NK cells to kill relevant targets, such as tumor or virally infected cells, depends on the delicate balance of the patterns of expression of MHC class I-specific inhibitory NK receptors and activating receptors.[30,31] As there is no animal model in which NK-cell activities are genetically and selectively deficient to rule out the function of NK cells in viral infection and tumor growth and metastasis, most studies have relied on depleting NK cells in mice using monoclonal or polyclonal antibodies.[32,33] Depletion of NK cells *in vivo* by anti-NK antibody leads to enhanced tumor formation in several mouse tumor models.[15,34] Therefore, the role of NK cells in the course of tumor growth and infiltration as well as viral infection remains one of the major topics in tumor immunology.

In the present study, we investigated the direct involvement of NK cells in growth and infiltration of PEL cells using T, B and NK knock-out NOG mice,[13,35,36] and T and B knock-out NOD/SCID mice. NK knock-out NOG mice were most efficient in the formation of large tumors, massive ascites and infiltration within 3 weeks in comparison with NK-bearing NOD/SCID mice. We also provide evidence that activated human NK cells inhibit tumor growth and infiltration in NOG mice. These results suggest that NK cells play an important role in tumor growth and infiltration, and activated NK cells could be a promising immunotherapeutic strategy against AIDS-associated PEL or other malignancies either alone or in combination with conventional therapy.

Materials and Methods

Mice and inoculation of cell lines. NOG and NOD/SCID mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions at the Animal Center of Tokyo Medical and Dental University (Tokyo, Japan). The Ethical Review Committee of the institute approved the experimental protocol.

Primary effusion lymphoma cell lines BCBL-1[37] and TY-1,[38] NK cell line KHYG-1 and bcr-abl$^+$ leukemic cell line K562 were cultured in RPMI-1640 medium supplemented with 2% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 100 U/mL penicillin and 10 μg/mL streptomycin. BCBL-1 and TY-1 cells were washed twice with serum-free RPMI-1640 and resuspended in fresh RPMI-1640. Mice were anesthetized with ether and cells were inoculated either subcutaneously (sc) in the postauricular region or intraperitoneally (ip) in the abdominal region of mice at doses of 1×10^7 and 2×10^6 cells per mouse, respectively. BCBL-1 cells were also inoculated either sc in the postauricular region or ip in the abdominal region of NOD/SCID mice with or without pretreatment with TMβ1 antibody, or in NOG mice. All mice were killed 3 weeks after inoculation with PEL cells. We measured tumor size, collected ascites from the abdomen of mice, and measured the volume of ascites.

Isolation and culture of NK cells. Blood was collected after obtaining informed consent from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated from the blood by Ficoll-Hypaque gradient centrifugation (Amersham Biosciences, Uppsala, Sweden), washed twice with RPMI-1640, and the number of cells counted. To generate activated NK cells, PBMC were cultured in anti-CD16-coated flasks with AIM-V medium (Invitrogen, Tokyo, Japan) supplemented with 5% auto-plasma, 700 U/mL interleukin (IL)-2 (Chiron, Amsterdam, the Netherlands), and 1 μL/mL OK432 (Chugai Pharmaceutical, Tokyo, Japan) for 24 h at 39°C, and then the cultured cells were centrifuged at 550 *g* for 10 min and the supernatants were discarded. Cells were again cultured in anti-CD16-uncoated flasks with AIM-V medium supplemented with 5% auto-plasma, and 700 U/mL IL-2 at 37°C for 2–3 weeks. During culture periods, we added medium several times for expansion and maintenance of activated NK cells. The purity of NK cells was 92–95%.

Flow cytometric analysis and cytotoxic activity. For five-color flow cytometric analysis (Cytomics FC500; Beckman Coulter, Miami, FL, USA), freshly isolated and activated NK cells were stained with monoclonal antibodies (ECD-labeled anti-CD3, PC5-labeled anti-CD4, PC7-labeled anti-CD8, PC7-labeled anti-CD16, PE or PC7-labeled anti-CD45, PC5-labeled anti-CD56, and PE-labeled anti-CD69 [Immunotech, Marseile, France]) and appropriate anti-isotypic monoclonal antibodies stained as negative controls. Data were analyzed by using CXP Analysis software version 1.1.

Freshly isolated and activated NK cells were tested for cytotoxic activity at various effector-to-target (E/T) ratios in a calcein-AM release assay using TERASCAN VP (Minerva Tech., Tokyo, Japan). We labeled the target cells with the immunofluorescent dye Calcein-AM solution (Do Jindo Laboratory, Kumamoto, Japan) and incubated them for 30 min. The cells were then washed with phosphate-buffer saline (PBS)(–) and the fluorescence intensity checked. Target cells and effector cells were suspended in RPMI-1640 with 10% FBS at various E/T ratios, added into 96-well plates and incubated for 2 h, and the fluorescence intensity was again checked.

Inoculation of activated NK cells into mice and collection of samples. Mice were inoculated with BCBL-1 (2×10^6) cells ip in the abdominal region of NOG mice. Three days after inoculation of PEL cells, mice were treated with either RPMI-1640 (control mice) or activated NK cells (1×10^7) ip on days 4, 10 and 17. All mice were killed 3 weeks after inoculation with PEL cells. We measured tumor size, collected ascites from the abdomen of mice, and measured the volume of ascites. Tissues and various organs of mice were also collected and fixed with 10% buffered formalin (Streck Tissue Fixative, Omaha, NE, USA), then processed to paraffin-embedded sections for staining with hematoxylin and eosin (HE) and immunostaining.

Immunohistochemistry. Paraffin sections of various organs were deparaffinized and hydrated in xylene or clearing agents and a graded alcohol series, then rinsed for 5 min in water. Deparaffinized samples were incubated with 0.025% trypsin/PBS for 30 min followed by washing, and then incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature before being washed twice with PBS. Immunostaining was done for PEL cells with a 1:500 dilution of primary rabbit polyclonal antibody specific for HHV-8-encoded LANA.[39] This was followed by washing in PBS and incubation with a secondary antibody, biotinylated antirabbit IgG, after which cells were again washed in PBS and incubated with horseradish peroxidase-conjugated streptavidin for 30 min at room temperature. After two washes in PBS, the amplification procedure was carried out using kits according to the manufacturer's instructions (catalyzed signal amplification system kit; DAKO, Copenhagen, Denmark). The signal was visualized using 0.2 mg/mL diaminobenzidine and 0.015% H_2O_2 in 0.05 M Tris-HCl, pH 7.6. Positive staining was visualized after incubation of these samples with a mixture of 0.05% 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl buffer and 0.01% H_2O_2 for 5 min. The samples were counterstained with hematoxylin for 2 min, dehydrated completely, cleaned in xylene and then mounted. HE and immunostaining were visualized and photographed under light microscopy (BX41 and DP70; Olympus, Tokyo, Japan).

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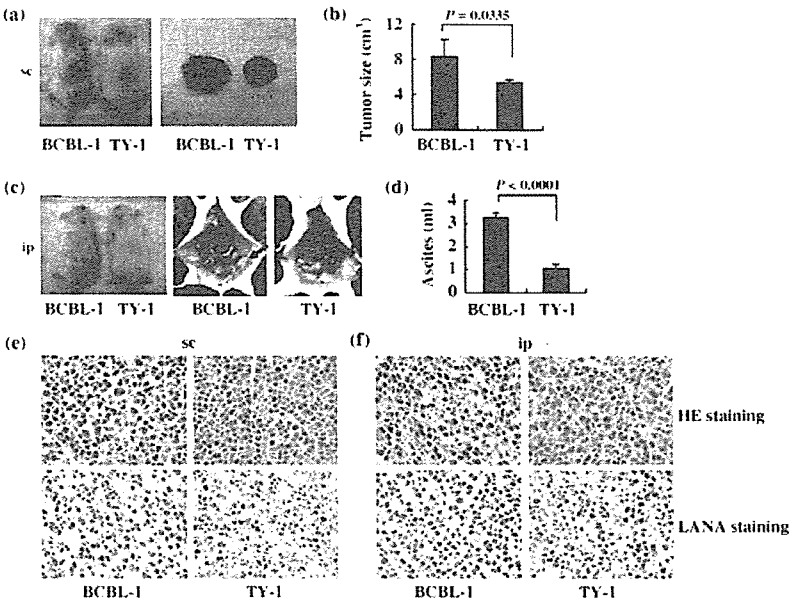

Fig. 1. Successful engraftment and tumor marker of primary effusion lymphoma (PEL) cells in T, B and natural killer (NK) knock-out NOG mice. (a) Photograph of mice inoculated with BCBL-1 and TY-1 cells subcutaneously in the postauricular region (left panel) and those of subcutaneously formed BCBL-1 and TY-1 tumor 3 weeks after inoculation of cells (right panel). (b) Subcutaneous tumor size of mice inoculated with BCBL-1 and TY-1 cells, shown as the mean ± s.e.m. from five mice ($P = 0.0335$). (c) Photograph of ascites-bearing mice inoculated with BCBL-1 and TY-1 cells intraperitonealy in the abdominal region (left panel) and peritoneal cavity of mice 21 days after inoculation of BCBL-1 (middle panel) and TY-1 cells (right panel). Arrow head indicates the tumor in mice inoculated intraperitonealy. (d) Volume of ascites in mice inoculated with various BCBL-1 and TY-1 cells, shown as the mean ± s.e.m. from five mice ($P < 0.0001$). (e,f) Hematoxylin–eosin (HE) and immunohistochemical staining of tumor tissue of BCBL-1 and TY-1 cells injected mice. Upper panels represent HE staining. Immunohistochemical staining was conducted using rabbit anti-LANA (lower panels). Left and right panels represent results with BCBL-1 and TY-1, respectively (magnification, ×40). Data are from (e) mice inoculated subcutaneously and (f) mice inoculated intraperitonealy.

Statistical analysis. The statistical analysis was carried out using StatView J-4.5 (Hulinks, Tokyo, Japan).

Results

Rapid tumor and massive ascites formation and infiltration of PEL cells in T, B and NK knock-out NOG mice. To investigate *in vivo* growth, PEL cell lines (BCBL-1 and TY-1) were inoculated sc in the postauricular region of NOG mice (Fig. 1a,b). Mice inoculated with cell lines BCBL-1 and TY-1 produced a visible tumor within 3 weeks in all NOG mice. The BCBL-1 cell line was very efficient in the formation of a large tumor (Fig. 1a,b), as well as development of clinical signs of near-death, such as pilorection, weight loss and cachexia in mice at the time of killing. The average tumor size in NOG mice inoculated with BCBL-1 and TY-1 was 8.25 cm^3 and 5.43 cm^3, respectively. PEL is an AIDS-associated non-Hodgkin's lymphoma that is characterized by lymphomatous effusions of serous cavities and rarely presents with a definable tumor mass.[5,7] To establish a clinically relevant PEL model, we inoculated BCBL-1 and TY-1 cells ip in the abdominal region of NOG mice (Fig. 1c,d). BCBL-1 and TY-1 produced massive ascites and a small tumor mass in the peritoneal cavity within 3 weeks of inoculation in all mice. The BCBL-1 cell line was most efficient in the formation of massive ascites (Fig. 1c,d), as well as development of clinical signs of near-death. The average volume of ascites in NOG mice inoculated with BCBL-1 and TY-1 was 3.26 mL and 1.05 mL, respectively. To test whether tumors maintain original histomorphology and expression patterns of tumor markers in NOG, we carried out HE and immunostaining of tumor tissues and various organs obtained from mice inoculated with BCBL-1 and TY-1 cells. Histological and immunological analysis revealed that *in vivo* tumor cells had well-preserved morphology as well as expression of the viral gene *LANA* (Fig. 1e,f). These results show that PEL cell lines inoculated either sc into the postauricular region or ip in the abdominal region of NOG mice were able to produce a large tumor and ascites very efficiently. Interestingly, ip-inoculated PEL cells were found to form clinically relevant lymphomatous effusions in the peritoneal cavity as well as a small definable mass.

To assess the tissue distribution of PEL cells, we carried out histological examinations of the different organs of NOG mice after inoculation of the cells. Infiltration of tumor cells was found not only in primary tumor tissues, but also to a lesser extent in the lung of NOG mice inoculated sc with BCBL-1 and TY-1 (Fig. 2a,b). We found that mice inoculated ip with BCBL-1 cells exhibited infiltration in the lung, liver and spleen (Fig. 2c), whereas TY-1 cells did so to a lesser extent only in the lung and liver (Fig. 2d). HE and immunohistochemical staining showed a degree of infiltration of tumor cells at the site of inoculation and various organs with BCBL-1 and TY-1 (Fig. 2). Furthermore, BCBL-1 was most efficient at infiltrating the lung (Fig. 2a,c). Interestingly, ip-inoculated PEL cells appeared to infiltrate various organs of mice more aggressively and massively than sc inoculation. This extremely rapid tumor formation and infiltration in all mice is one of the hallmarks of our clinically relevant animal model without changes in histomorphology or tumor marker expression.

Role of NK cells in the growth and infiltration of PEL cell *in vivo*. Severe combined immunodeficiency mice lack functional T and B lymphocytes, but NK-cell activity remains normal.[10,17,40] Despite severe immunological defects, SCID mice have the ability to reject xenografts. Further, immunosupression of SCID by treatment with etoposide, irradiation or an anti-NK antibody, which transiently abrogates NK-cell activity *in vivo*, results in enhanced tumor growth in mice.[41–47] To determine the possibility that NK-cell activity suppresses tumorigenesis in conventional SCID mice, the PEL cell line BCBL-1 was inoculated either sc in the postauricular region or ip in the abdominal region of T and B knock-out NOD/SCID mice with or without pretreatment of with TMβ1 antibody, or T, B and NK knock-out NOG mice (Fig. 3a–g). BCBL-1 cells were able to produce tumors at inoculation sites in NOD/SCID mice with common γ-chain. Immunosupression of NOD/SCID by treatment with an antimurine TMβ1 antibody, which transiently abrogates natural killer cell activity *in vivo*, resulted in induction of larger tumor and ascites formation in comparison with non-treated NOD/SCID mice. NOG mice lacking common γ-chain inoculated with BCBL-1 cells were most efficient in the formation of large tumor and massive ascites within 3 weeks.