表 2 Rituxan 治療 1 か月後の各種検査値異常の変化 (n=20)

	治療後の変化	p 値
ヘモグロビン (g/dl)	+4.8	<0.001
血小板 (×10E4/ul)	+7.5	0.011
アルブミン (g/dl)	+0.9	<0.001
CRP (g/dl)	−5.9	0.003
CD4 数 (/ul)	+80	0.011
血漿 HHV-8 (copies/ml)	−700	0.018

文献 17) より改変

ある。確定診断は，腫大リンパ節あるいは節外病変の生検を行い，病理学的検討を行うことでなされる。CRP の高値や血清 HHV-8 のウイルス量の上昇，血小板減少などの検査値も本疾患を疑う手がかりとなる[21]。生検病理では大部分が形質細胞型であり，硝子血管型は 10% 未満である。HIV 患者では，全例で病変内に HHV-8 が証明される[3]。

重要なのは，HHV-8 の活性化が病態の本態であるため，HHV-8 関連疾患，特にリンパ腫の存在を見逃さないことである。特に HIV 合併 MCD では悪性度の高い形質芽球性リンパ腫の発症リスクが高い[22]。その他，PEL や稀ではあるが，geminotropic lymphoproliferative disorder と呼ばれるリンパ増殖疾患の合併も報告されている[23]。MCD，形質芽球性リンパ腫は HHV-8 単独で起こるが，PEL や geminotropic lymphoproliferative disorder の発症には，EBV の共感染が関連していることが多い。HIV 合併 MCD から発生した形質芽球性リンパ腫は monoclonal な増殖である[24]。恐らくは HHV-8 の活性化によって引き起こされた，HHV-8 感染 immunoblasts の過剰な増殖により，新たな変異の蓄積が起こることが発症に関連すると推測されている。MCD 患者 60 例を中央値で 20 か月追跡したプロスペクティブコホートでは，14 例が非ホジキンリンパ腫を発症した (3 例：PEL，5 例：PEL-like phenotype EBV-HHV-8 リンパ腫，6 例：形質芽球性リンパ腫/白血病)。非ホジキンリンパ腫の発生頻度は MCD を発症していない HIV 患者の 15 倍高いと推定された[25]。

検討症例は少ないが，2 報 6 例で MCD ではガリウムシンチで取り込みが見られないとする報告があり[26,27]，高度に集積が見られる合併悪性リンパ腫の検出において有用である可能性がある。

HIV 合併 MCD における FDG-PET での検討では，再燃例を含む活動性 MCD7 例と寛解期 2 例について行い，CT で腫大の見られないリンパ節での集積も検出され病変リンパ節の検出感度が高かったという報告がある[28]。SUV 値は活動期に比べ寛解期で有意に低値であり (Median 4.8 vs 2.5，p=0.011)，活動性の評価や治療の効果判定の指標となりうる可能性も示唆された。ただし，悪性リンパ腫合併との鑑別は難しく，MCD 診断における本検査の意義は現時点では不明である。

腫瘍性疾患の他にも，多発神経炎や重症筋無力症などの神経系合併症を起こすことがある。中枢神経系に MCD の病変を形成した報告もある[29]。

7. 治　療

現時点では，治療例に関するケースレポートや少数例での検討があるのみであり，各治療法を比較検討したものはなく，標準治療は存在しない。ただし Rituximab 単独，あるいは化学療法と組み合わせた治療法で，有効かつ長期にわたる寛解状態を誘導したという報告が多く，今後のさらなる検討が期待されている。

1) Rituximab (抗 CD20 抗体) と化学療法の併用，あるいは単独治療

Rituximab が単独，および化学療法との併用で有効であったとの報告が多数存在する。特に化学療法との併用例では，寛解持続期間が長く，検討症例数の点でも他の治療法に比べて信頼性の高い報告がなされていると言える。一方で，MCD に高頻度に合併する KS については，Rituximab の投与により悪化する可能性が示唆されている。Rituximab の投与後の再燃例においても，Rituximab の単剤再治療が有効であり，耐性を誘導されることなく反復治療が可能である可能性も示唆されている。

一方，多剤併用化学療法あるいは単剤での治療例では，効果は持続的でなく再燃を防ぐために維持治療を要するとする報告が多い。

i) Rituximab と化学療法との併用

① 4 例の HIV 合併 MCD に対し，Rituximab 4 回投与と化学療法を併用することにより，16-46 か月という長期にわたって寛解が維持できた[30] (表 3)。

② 化学療法で寛解状態になったが，維持治療を中止すると再燃するために，化学療法を中止できない HIV 合併 MCD 23 例を対象。vinblastine, etoposide あるいは，peglated liposomal doxorubicin を使用して，中央値で 13 か月間寛解状態を維持していた。Rituximab を 1 週毎に計 4 回投与し，化学療法を中止したところ，22 例で 60 日間，17 例で 1 年間，再発無く寛解状態を維持できた (1 例は他の原因で死亡)。KS の合併があった 12 例中 8 例では悪化が見られた[31]。

③ 硝子血管型の HHV-8 陽性 HIV 合併 MCD 症例に対し，HAART と同時に R-CHOP を 6 コース行い，寛解となった。3 年間の経過観察で再燃なし[32]。

表 3 Rituximab と化学療法の併用療法による長期寛解例

	Case 1	Case 2	Case 3	Case 4
CHOP（cycle）	3	1	0	1
Rituximab（dose）	4	4	4	4
その他	・liposomal doxorubicin ・Vincristine	Dexamethasone	・Dexamethasone ・liposomal doxorubicin	none
寛解期間（月）	23	4	42	16
再発の有無	No（肺 KS で死亡）	Yes	No	No
再発時の治療	No	CHOP×1 ＋Rituximab×4	NA	NA
寛解期間 2（月）	NA	46	NA	NA

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ii） Rituximab 単独

① HIV 合併 MCD 症例に対する Rituximab での症例報告 10 例のまとめ。2 例は死亡したが 8 例（80％）は CR となっていた。全例 HAART 施行中の発症であった。CR 例 8 例のうち 5 例は KS の合併を認めたが，うち 3 例（60％）では KS 病変の悪化を認めた[33]。

② 治療歴のない 21 例の HIV 合併 MCD に対し，1 週毎に 4 回，Rituximab を投与し，中央値で 12 か月間経過観察した。1 例は治療完遂前に死亡。治療を完遂できた 20 例の全例で発熱などの症状が消失した。2 年間の全生存率は 95％で，再発なし生存は 79％であった。診断時には 80％で血中の HHV-8 が検出されたが，治療 1 か月後には 20％，3 か月後には 10％で検出された。KS 合併例 11 例中 4 例で病変の悪化を認めた[34]。

③ ABV（doxorubicin, vincristine, bleomycin）8 サイクル，抗 IL-6 抗体，cidofovir, liposomal daunorubicin 24 コースのいずれの治療も無効であった症例に対し，Rituximab の単回投与が奏功して HHV-8 の DNA が検出限界以下になり，その後 14 か月にわたり寛解が維持できた[35]。

④ Rituximab で治療され，寛解後再発した 3 例に対し，Rituximab 単剤による再治療が有効であった。反応はウイルス量と相関していた[36]。

iii） 化学療法単独

① エトポシド 50 mg/day による治療の 2 例。1 例は初回治療，2 例目はドキシル，パクリタキセル，経口ガンシクロビルによる治療にも関わらず再発を繰り返している例であった。2 例とも効果は持続的で，それぞれ 1.5 か月，6 か月観察しているが効果は持続している[37]。

② 非 HIV 症例で，腎疾患のためにシクロスポリン A の投与を 17 年受け，KS とキャッスルマン病を発生した症例。liposomal doxorubicin による治療で両者とも軽快。血清中の HHV-8 のウイルス量も減少が見られた[38]。

③ HIV 合併 MCD 症例で，liposomal doxyrubicin とエトポシドが有効であった 1 症例[39]。

④ 非 HIV 症例の HHV-8 関連 MCD に対して，MTX が有効であり，54 か月以上に渡って寛解を持続していた[40]。

2） 抗ウイルス薬

HHV-8 は in vitro でガンシクロビル，ホスカビル，シドフォビルのいずれにも有効であり，特にシドフォビルは最も強力な抗 HHV-8 活性を持っている[41]。

臨床例で有効性が報告されているのは，現時点ではガンシクロビルのみであり，シドフォビルについては無効であったという報告がある[42]。

i） ガンシクロビルの抗 HHV-8 作用[43]

唾液中に HHV-8 の排出が間欠的に認められている 26 症例（HIV 感染 16 例と非 HIV 10 例）を対象とした randomized, double-blind, placebo-controlled, crossover trial の報告がある。まず被験者はバルガンシクロビル 900 mg/day 投与群とプラセボ投与群 13 例ずつに割り付けられ，8 週間に渡って内服した。その後 2 週間の washout の期間をおいた後，プラセボとバルガンシクロビルにそれぞれ内服を切り替え同じく 8 週間の内服した。試験期間中，被験者は連日，口腔内より唾液のサンプルを採取し real time PCR により HHV-8 の定量を行った。検体の回収率は 88％であった。その結果，HHV-8 が検出される期間はバラガンシクロビル投与により 46％も減少し，検出された検体のウイルス量は 0.3 log 低く，いずれも有意差が認められ，ガンシクロビルの抗 HHV-8 作用が示された。治療開始前に HHV-8 が検出可能であった症例では，HIV 感染者と非 HIV 感染者ともに，バルガンシクロビルの 8 週投与により 2 log

表 4 Valganciclovir によるウイルス排出期間の変化

	HIV 感染	非 HIV 感染
>20% 減少	11 (68.8%)	5 (55.5%)
±20% 以内	2 (12.5%)	3 (33.3%)
>20% 増加	3 (18.8%)	1 (11.1%)
合計	16 例	9 例*

*1 例は全期間でウイルスの排出を認めず，解析から除外した
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copies/ml 程度のウイルス量の減少が見られた。

ところがバルガンシクロビルの抗 HHV-8 効果は個体差が存在する可能性がある。表 4 に Valganciclovir 投与によるウイルス HHV-8 排出期間の変化をまとめたが，減少が見られたのは HIV 症例の 7 割弱であり，2 割弱の症例では逆にウイルス排出期間の延長が認められている。

ii） ガンシクロビル単剤による有効例

ガンシクロビルによる治療が有効であった HIV 合併 MCD3 例の報告がある。1 例は他の原因で死亡したが，1 例は治療量を 7 日間と維持量 14 日間の治療を行い，以後は再燃なく 12 か月が経過した。もう 1 例は，4 週間程度のガンシクロビルによる間歇的治療を繰り返し，1 年 2 か月で 4 回の再燃を繰り返したが，その都度ガンシクロビルが有効であった。最後の再燃時に HAART が導入され，以後は 18 か月にわたり再燃を認めていない[44]。

3） その他

i） サリドマイド：Rituximab とサリドマイドの併用治療により 20 か月以上寛解を維持している症例[45]や，40 か月に渡りサリドマイドを単剤維持投与され，寛解状態を維持しているという報告がある[46]。liposomal doxorubicin を含む各種多剤併用療法が無効であったが，経口エトポシドがある程度の有効性を示し，その後サリドマイドにスイッチすることで血小板数が増加し，著効した症例報告もある[47]。

ii） 抗 IL-6 レセプター抗体：他施設共同，前向き試験が 28 例で検討され，有望な成績が出されている[48]。しかし本試験の対象症例には HIV 陽性例は含まれておらず，HHV-8 陽性例も 2 例しか含まれていない。

iii） インターフェロン α：HIV 合併 MCD に対し，3 例中 2 例で 3–6 か月の寛解が得られた[3]。ケースレポートでは単独治療で 1 年以上[49]，あるいは 2 年以上[50]にわたり寛解状態が維持できたという報告がある。

iv） 脾臓摘出：効果は 1–3 か月程度でしかなく，また再燃のための治療を要する[3]。

v） 多剤併用治療：Rituximab による治療が無効であり，多臓器不全から一時は心停止に到った症例が，その後，高用量ステロイド，ビンクリスチン，ブレオマイシン，ガンシクロビルの併用療法により寛解に持ち込んだ症例[51]。

vi） ステロイド：しばしば全身的治療として他の治療と併用されるが，単独で持続的な効果が証明された報告は見あたらない。

8. MCD における HAART の影響

先述のように，HAART 導入後に MCD の発生頻度は逆に増加しているという疫学的事実も合わせ，HAART による免疫の再構築が MCD の予後に与える影響については不明な点が多い。

HAART の時代になって MCD の予後が改善したという報告がある[52]。しかしながら，1）無症状で血中 HHV-8 が検出されていた症例が，HAART 後に結局 MCD を発症した[53]という報告や，2）MCD の症例に HAART を導入しても，再燃を抑える効果は認められなかったという報告[52]，3）HAART 導入後も，血中の HHV-8 レベルは不変であり，HAART 開始 5 か月後に MCD，16 か月後に PEL，23 か月後に肺カポジ肉腫を発症して死亡した症例[1]など，HAART による免疫再構築でも，MCD のコントロールに必要な抗 HHV-8 免疫を，短期間で誘導することは難しいことが示唆されている。

一方で，HAART 導入後に免疫再構築症候群（IRIS）のような機序で，MCD の発症リスクが高まるという報告もなされている[20]。以上のことから，Stebbing らは MCD に関する総説の中で，「HAART 導入前に MCD を発症した患者については，HAART 導入前に Rituximab による治療を行う。その後 HAART により免疫が再構築されるまで，HHV-8 の増殖を抑える目的で 3 か月程度のバルガンシクロビルの投与を検討する」という考え方を述べている[54]。

文　献

1） Castleman B, Iverson L, Menendez VP : Localized mediastinal lymphnode hyperplasia resembling thymoma. Cancer 9 (4) : 822–830, 1956.

2） Larroche C, Cacoub P, Soulier J, et al. : Castleman's disease and lymphoma : report of eight cases in HIV-negative patients and literature review. Am J Hematol 69 (2) : 119–126, 2002.

3） Oksenhendler E, Duarte M, Soulier J, et al. : Multicentric Castleman's disease in HIV infection : a clinical and pathological study of 20 patients. AIDS 10 (1) : 61–67, 1996.

4） Powles T, Stebbing J, Bazeos A, et al. : The role of immune suppression and HHV-8 in the increasing incidence

of HIV-associated multicentric Castleman's disease. Ann Oncol 20 (4) : 775-779, 2009.

5) Casper C : The aetiology and management of Castleman disease at 50 years : translating pathophysiology to patient care. Br J Haematol 129 (1) : 3-17, 2005.

6) Gaba AR, Stein RS, Sweet DL, et al. : Multicentric giant lymph node hyperplasia. Am J Clin Pathol 69 (1) : 86-90, 1978.

7) Yamasaki S, Iino T, Nakamura M, et al. : Detection of human herpesvirus-8 in peripheral blood mononuclear cells from adult Japanese patients with multicentric Castleman's disease. Br J Haematol 120 (3) : 471-477, 2003.

8) Suda T, Katano H, Delsol G, et al. : HHV-8 infection status of AIDS-unrelated and AIDS-associated multicentric Castleman's disease. Pathol Int 51 (9) : 671-679, 2001.

9) Codish S, Abu-Shakra M, Ariad S, et al. : Manifestations of three HHV-8-related diseases in an HIV-negative patient : immunoblastic variant multicentric Castleman's disease, primary effusion lymphoma, and Kaposi's sarcoma. Am J Hematol 65 (4) : 310-314, 2000.

10) Minami R, Yamamoto M, Takahama S, et al. : Human herpesvirus 8 DNA load in the leukocytes correlates with the platelet counts in HIV type 1-infected individuals. AIDS Res Hum Retroviruses 25 (1) : 1-8, 2009.

11) Bestawros A, Boulassel MR, Michel RP, et al. : HHV-8 linked to Kaposi's sarcoma, Castleman's disease and primary effusion lymphoma in a HIV-1-infected man. J Clin Virol 42 (2) : 179-181, 2008.

12) Yoshizaki K, Matsuda T, Nishimoto N, et al. : Pathogenic significance of interleukin-6 (IL-6/BSF-2) in Castleman's disease. Blood 74 (4) : 1360-1367, 1989.

13) Cannon JS, Nicholas J, Orenstein JM, et al. : Heterogeneity of viral IL-6 expression in HHV-8-associated diseases. J Infect Dis 180 (3) : 824-828, 1999.

14) Foussat A, Fior R, Girard T, et al. : Involvement of human interleukin-6 in systemic manifestations of human herpesvirus type 8-associated multicentric Castleman's disease. AIDS 13 (1) : 150-152, 1999.

15) Du MQ, Liu H, Diss TC, et al. : Kaposi sarcoma-associated herpesvirus infects monotypic (IgM lambda) but polyclonal naive B cells in Castleman disease and associated lymphoproliferative disorders. Blood 97 (7) : 2130-2136, 2001.

16) Judde JG, Lacoste V, Briere J, et al. : Monoclonality or oligoclonality of human herpesvirus 8 terminal repeat sequences in Kaposi's sarcoma and other diseases. J Natl Cancer Inst 92 (9) : 729-736, 2000.

17) Bower M, Powles T, Williams S, et al. : Brief communication : rituximab in HIV-associated multicentric Castleman disease. Ann Intern Med 147 (12) : 836-839, 2007.

18) Boivin G, Cote S, Cloutier N, et al. : Quantification of human herpesvirus 8 by real-time PCR in blood fractions of AIDS patients with Kaposi's sarcoma and multicentric Castleman's disease. J Med Virol 68 (3) : 399-403, 2002.

19) Grandadam M, Dupin N, Calvez V, et al. : Exacerbations of clinical symptoms in human immunodeficiency virus type 1-infected patients with multicentric Castleman's disease are associated with a high increase in Kaposi's sarcoma herpesvirus DNA load in peripheral blood mononuclear cells. J Infect Dis 175 (5) : 1198-1201, 1997.

20) Zietz C, Bogner JR, Goebel FD, et al. : An unusual cluster of cases of Castleman's disease during highly active antiretroviral therapy for AIDS. N Engl J Med 340 (24) : 1923-1924, 1999.

21) Stebbing J, Pantanowitz L, Dayyani F, et al. : HIV-associated multicentric Castleman's disease. Am J Hematol 83 (6) : 498-503, 2008.

22) Deloose ST, Smit LA, Pals FT, et al. : High incidence of Kaposi sarcoma-associated herpesvirus infection in HIV-related solid immunoblastic/plasmablastic diffuse large B-cell lymphoma. Leukemia 19 (5) : 851-855, 2005.

23) Du MQ, Diss TC, Liu H, et al. : KSHV- and EBV-associated germinotropic lymphoproliferative disorder. Blood 100 (9) : 3415-3418, 2002.

24) Dupin N, Diss TL, Kellam P, et al. : HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. Blood 95 (4) : 1406-1412, 2000.

25) Oksenhendler E, Boulanger E, Galicier L, et al. : High incidence of Kaposi sarcoma-associated herpesvirus-related non-Hodgkin lymphoma in patients with HIV infection and multicentric Castleman disease. Blood 99 (7) : 2331-2336, 2002.

26) Chim CS, Choi FP, Ooi GC, et al. : Absence of gallium uptake in multicentric Castleman's disease of plasma cell type. Haematologica 86 (4) : 442-443, 2001.

27) Okamoto I, Iyonaga K, Fujii K, et al. : Absence of gallium uptake in unicentric and multicentric Castleman's disease. Intern Med 42 (8) : 735-739, 2003.

28) Barker R, Kazmi F, Stebbing J, et al. : FDG-PET/CT imaging in the management of HIV-associated multicentric Castleman's disease. Eur J Nucl Med Mol Imaging 36 (4) : 648-652, 2009.

29) Ribeiro LT, Simao GN, Matos AL, et al. : Intracranial Castleman's disease presenting as hypopituitarism. Neuroradiology 46 (10) : 830-833, 2004.

30) Bestawros A, Michel R, Seguin C, et al. : Multicentric Castleman's disease treated with combination chemotherapy and rituximab in four HIV-positive men : a case series. Am J Hematol 83 (6) : 508-511, 2008.

31) Gerard L, Berezne A, Galicier L, et al. : Prospective study of rituximab in chemotherapy-dependent human immunodeficiency virus associated multicentric Castleman's disease : ANRS 117 CastlemaB Trial. J Clin Oncol 25 (22) : 3350-3356, 2007.

32) Flejsierowicz M, Ahmed MS, Kotov P, et al. : Successful treatment of aggressive HIV-associated multicentric Castleman's disease : a case report. WMJ 107 (4) : 191-194, 2008.

33) Casquero A, Barroso A, Fernandez Guerrero ML, et al. : Use of rituximab as a salvage therapy for HIV-associated multicentric Castleman disease. Ann Hematol 85 (3) : 185-187, 2006.

34) Bower M, Powles T, Williams S, et al. : Brief communication : rituximab in HIV-associated multicentric Castleman disease. Ann Intern Med 147 (12) : 836-839, 2007.

35) Corbellino M, Bestetti G, Scalamogna C, et al. : Long-term remission of Kaposi sarcoma-associated herpesvirus-related multicentric Castleman disease with anti-CD20 monoclonal antibody therapy. Blood 98 (12) : 3473-3475, 2001.

36) Powles T, Stebbing J, Montoto S, et al. : Rituximab as retreatment for rituximab pretreated HIV-associated multicentric Castleman disease. Blood 110 (12) : 4132-4133, 2007.

37) Scott D, Cabral L, Harrington WJ Jr., et al. : Treatment of HIV-associated multicentric Castleman's disease with oral etoposide. Am J Hematol 66 (2) : 148-150, 2001.

38) Bollen J, Polstra A, Van Der Kuyl A, et al. : Multicentric Castleman's disease and Kaposi's sarcoma in a cyclosporin treated, HIV-1 negative patient : case report. BMC Blood Disord 3 (1) : 3, 2003.

39) Fowler A, Collins L, de Ruiter A, et al. : Multicentric Castleman's disease in a patient with HIV. Int J STD AIDS 17 (1) : 63-64, 2006.

40) Jacobs SA, Vidnovic N, Patel H, et al. : Durable remission of HIV-negative, Kaposi's sarcoma herpes virus-associated multicentric Castleman disease in patient with rheumatoid arthritis treated with methotrexate. Clin Rheumatol 26 (7) : 1148-1150, 2007.

41) Kedes DH, Ganem D : Sensitivity of Kaposi's sarcoma-associated herpesvirus replication to antiviral drugs. Implications for potential therapy. J Clin Invest 99 (9) : 2082-2086, 1997.

42) Berezne A, Agbalika F, Oksenhendler E : Failure of cidofovir in HIV-associated multicentric Castleman disease. Blood 103 (11) : 4368-4369, 2004.

43) Casper C, Krantz EM, Corey L, et al. : Valganciclovir for suppression of human herpesvirus-8 replication : a randomized, double-blind, placebo-controlled, crossover trial. J Infect Dis 198 (1) : 23-30, 2008.

44) Casper C, Nichols WG, Huang ML, et al. : Remission of HHV-8 and HIV-associated multicentric Castleman disease with ganciclovir treatment. Blood 103 (5) : 1632-1634, 2004.

45) Stary G, Kohrgruber N, Herneth AM, et al. : Complete regression of HIV-associated multicentric Castleman disease treated with rituximab and thalidomide. AIDS 22 (10) : 1232-1234, 2008.

46) Starkey CR, Joste NE, Lee FC : Near-total resolution of multicentric Castleman disease by prolonged treatment with thalidomide. Am J Hematol 81 (4) : 303-304, 2006.

47) Jung CP, Emmerich B, Goebel FD, et al. : Successful treatment of a patient with HIV-associated multicentric Castleman disease (MCD) with thalidomide. Am J Hematol 75 (3) : 176-177, 2004.

48) Nishimoto N, Kanakura Y, Aozasa K, et al. : Humanized anti-interleukin-6 receptor antibody treatment of multicentric Castleman disease. Blood 106 (8) : 2627-2632, 2005.

49) Nord JA, Karter D : Low dose interferon-alpha therapy for HIV-associated multicentric Castleman's disease. Int J STD AIDS 14 (1) : 61-62, 2003.

50) Kumari P, Schechter GP, Saini N, Benator DA : Successful treatment of human immunodeficiency virus-related Castleman's disease with interferon-alpha. Clin Infect Dis 31 (2) : 602-604, 2000.

51) Buchler T, Dubash S, Lee V, et al. : Rituximab failure in fulminant multicentric HIV/human herpesvirus 8-associated Castleman's disease with multiorgan failure : report of two cases. AIDS 22 (13) : 1685-1687, 2008.

52) Aaron L, Lidove O, Yousry C, et al. : Human herpesvirus 8-positive Castleman disease in human immunodeficiency virus-infected patients : the impact of highly active antiretroviral therapy. Clin Infect Dis 35 (7) : 880-882, 2002.

53) de Jong RB, Kluin PM, Rosati S, et al. : Sustained high levels of serum HHV-8 DNA years before multicentric Castleman's disease despite full suppression of HIV with highly active antiretroviral therapy. AIDS 17 (9) : 1407-1408, 2003.

54) Stebbing J, Pantanowitz L, Dayyani F, et al. : HIV-associated multicentric Castleman's disease. Am J Hematol 83 (6) : 498-503, 2008.

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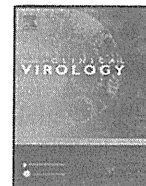

Evidence of inability of human cytomegalovirus to reactivate Kaposi's sarcoma-associated herpesvirus from latency in body cavity-based lymphocytes

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ABSTRACT

Background: Kaposi's sarcoma-associated herpesvirus (KSHV; also known as human herpesvirus 8 (HHV-8)) has been determined to be the most frequent cause of malignancies in AIDS patients. It is associated primarily with Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL), as well as with multicentric Castleman's disease (MCD).[2] The switch from the latent to the lytic stage is important in the maintenance of malignancy and viral infection. So far, the mechanism of its reactivation has not been fully understood.
Objectives: Human cytomegalovirus (HCMV) and KSHV might infect the same cells, and it was found by other groups that several viruses could reactivate KSHV from latency. We investigate whether HCMV infection could reactivate KSHV from latency in body cavity-based lymphocyte (BCBL-1) cells.
Study design and results: Laboratory strains of HCMV cannot infect B cells. In this article, we demonstrate that the UL131-repaired HCMV (vDW215-BADrUL131) derived from AD169 strain is able to infect B lymphocytes. We directly infected KSHV latent cells including BCBL-1 with vDW215-BADrUL131 to evaluate the ability of HCMV to reactivate KSHV. Inconsistent with previous reports in human fibroblast cells, our results provide direct evidence that HCMV is unable to reactivate KSHV from latency-to-lytic infection in BCBL-1 cell lines. As a control, herpes simplex virus type 1 (HSV-1) was shown to be able to reactivate KSHV.
Conclusions: Our observations, different from others, suggest that reactivation mechanisms for KSHV might vary in different cells.

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1. Background and objectives

Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8, also known as HHV-8) is the most frequent cause of malignancy in AIDS patients. This newly identified virus is associated with Kaposi's sarcoma and several B-cell malignancies such as primary effusion lymphoma as well as multicentric Castleman's disease (MCD).[2,6,7,19,25] KSHV infection in permissive cells including endothelial and fibroblast cells experiences a burst of lytic gene expression at a very early stage and subsequently causes a latent infection that can switch to a lytic infection upon reactivation.[4,13,26] Latent infection persists in a majority of cells, and only a small percentage of latent infection can be reactivated to the lytic cycle.[8] KSHV can successfully infect monocytes, endothelial/spindle cells, B cells and epithelial cells. The virus replicates primarily in KSHV-infected B cells and spindle cells.[5] Earlier studies have revealed that the KSHV latency-to-lytic switch is important in viral pathogenesis, secondary infection to maintain the amount of infected cells and tumourigenesis.[17] Information regarding reactivation of KSHV latency is mostly from *in vitro* reactivation by using chemicals or changing environment (such as hypoxia) or transfect-activating genes including RTA (replication transcriptional activator) and so on.

Herpesviruses including HCMV and KSHV are ubiquitous, and HCMV infects large populations worldwide, and exists latently in the infected host. Indeed, KSHV and HCMV are often detected simultaneously in the same patient; more importantly, both viruses can be isolated from blood cells.[3,9,12,15,16,22,29] HCMV IE1 can inhibit histone deacetylase (HDAC) activity,[21,27] which mimics the function of trichostatin A (TSA) or sodium butyrate (NaB); it is reasonable to speculate that HCMV can cause the lytic switch of KSHV infection if HCMV infected the cells that harbour the KSHV genome. Clinical data so far are still ambiguous regarding whether the mixed infection could result in KSHV reactivation. Recent studies showed

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that Tat of human immunodeficiency virus (HIV) could activate KSHV lytic infection through JAK/STAT signalling and that co-culture of HHV-6-infected T cells with KSHV latent B cells resulted in KSHV reactivation.[14,34] Vieira et al. clearly showed that an HCMV laboratory strain (AD169) of infection in established KSHV harboured a human fibroblast (HFF) cell line that can reactivate KSHV to produce viral particles,[30] and, more recently, the group mapped that UL112/113 is the viral component responsible for the reactivation of KSHV in HFF.[33] It is necessary to know whether HCMV can reactivate KSHV in BCBL-1 cells—our objective in the current studies.

2. Study design and results

HCMV laboratory strains (AD169 and Towne) lost their infectivity other than in human fibroblast cells due to mutation in the gene locus of UL131-128.[10] AD169 has one nucleotide insertion in UL131 that causes an amino acid (aa) frame shift and a functional defect of UL131. The Towne strain has an aa frame shift in UL130 and also causes defective tropism to other cells.[1,20] Clinical (wild) strains of HCMV might infect a wide range of cells. Fibroblast, endothelial, epithelial and blood cells are all susceptible to HCMV infection; it was reported that B cells isolated from 40% of the patients with active HCMV infection have viral DNA.[11] Remarkably, clinical (wild) strains of HCMV isolated from patients and then propagated in fibroblasts for just a few passages lose their ability to infect any cells other than the fibroblast cells. The fact that laboratory and clinical (wild) strains of HCMV are not equally infectious also serves to emphasise the difficulties inherent in the study of HCMV pathogenesis. The hypothesis that UL131-128 should be the determinant of cell tropism and that mutation of the gene locus is the mechanism by which HCMV loses its tropism to many cells in the laboratory was supported by recent molecular studies of HCMV tropisms.[31,32] In the studies, the investigator removed one nucleotide (nt) insertion of UL131 based on AD169. Due to the recovery of the mutation, the repaired AD169, namely vDW215-BADrUL131, was found to be able to infect not only fibroblast cells, but also endothelial and epithelial cells. Therefore, after repair of the mutations, the viral tropism can be recovered. However, it is still unknown whether the repaired HCMV could infect blood cells such as B lymphocytes.

First, we infected B lymphocytes (BJAB, B lymphocyte without KSHV latency; and BCBL-1, B lymphocyte with KSHV latency) and human fibroblast cells (Mrc-5 and HFF) with a laboratory strain of HCMV (AD169) at a multiplicity of infection (MOI) of 5 for 72 h; the whole-cell lysates were then applied to run polyacrylamide gel elctrophoresis (PAGE). Finally, we performed a Western blot to detect HCMV proteins. Compared with the infection of HCMV in human fibroblast cells (in which HCMV can express viral proteins as seen in IE1/2, MCP and pp28), no viral protein could be detected in BJAB or BCBL-1 cells (Fig. 1A). We subsequently infected BCBL-1 and BJAB cells with vDW215-BADrUL131, a repaired HCMV, at an MOI of 5 for different times. As can be seen in Fig. 1B, viral proteins from different stages can be detected. Both MCP and pp28 (UL99) are late proteins; pp28 (also called true late gene) production was proved to be DNA-replication dependent; such production suggests that DNA replication occurred as well. Therefore, the production of pp28 implies a successful infection of vDW215-BADrUL131 in B lymphocytes. Finally, we performed the plaque formation unit (PFU) assay. First, we infected BCBL-1 with HCMV AD169 or vDW215-BADrUL131 at MOI of 1 for different days as indicated in Fig. 1C. The medium and cells were collected and viral particles were released from cells by thaw and freeze for three cycles. After centrifugation, the supernatants were used to infect human fibroblast cells for counting viral plaques. The results in Fig. 1C show that vDW215-BADrUL131 can productively infect BCBL-1 cells while AD169 failed to produce any viral particles. Taken together, the data here provided evidence that HCMV is able to infect B lymphocyte after the tropism is recovered from AD169, and the presence of KSHV has no effect on the infection.

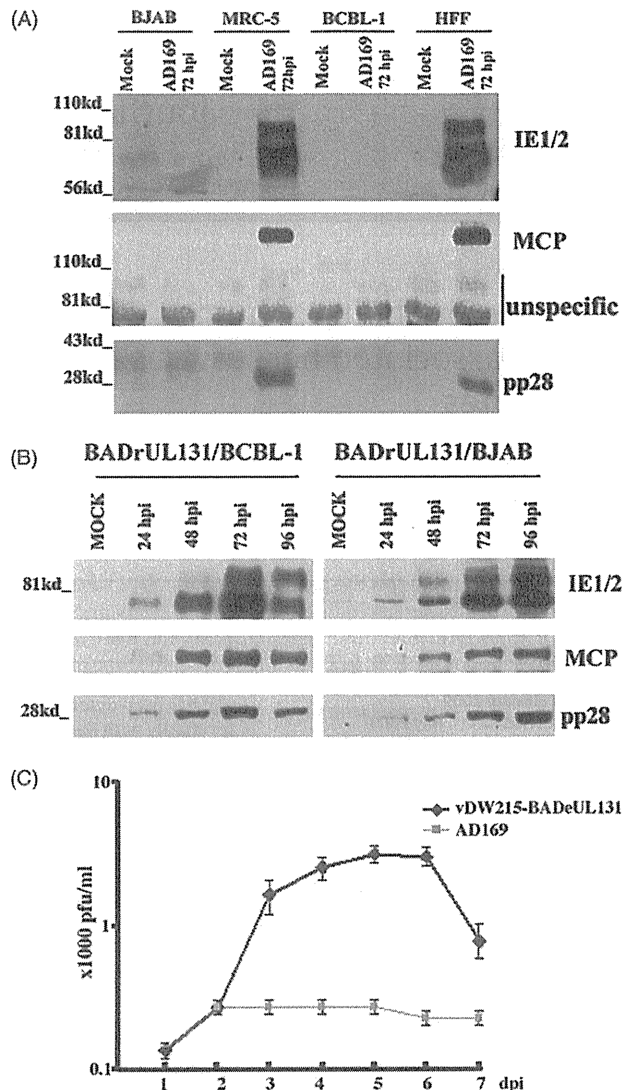

Fig. 1. Infectivity of laboratory strain and UL131-repaired HCMV in B cells. (A) After infection of laboratory strains of HMCV in cells for the time as indicated, cells were collected and lysed in Lamile buffer and applied in 7.5% PAGE; the viral proteins were detected with Western blot assay. (B) UL131-repaired HCMV, vDW215-BADrUL131 infection in B cells were detected by Western blot assay. (C) BCBL-1 cells in 12-well plates were infected with HCMV AD169 or vDW215-BADrUL131 at MOI of 1 and the medium was changed at 12 h after infection. The cells and medium was collected as the days post infection as indicated and the viral particles were released by thaw and freeze for three cycles. After centrifugation, the supernatant were applied for PFU assay. Data from triplicates were obtained and statistically analyzed; the error bars stand for the standard error from the three independent experiments.

Although most of those individuals with latently infected KSHV never suffer from any KSHV-associated diseases, such individuals run a risk of developing KS, PEL or MCD, with the greatest risk factor for these conditions being immunodeficiency, which occurs in AIDS patients, transplant recipients and patients with viral infections. The HCMV genome encodes some products that have immunomodulatory effects that can interfere with the host's immune system. In addition, the above observation demonstrates the fact B lymphocytes can be infected by HCMV as well as by KSHV.

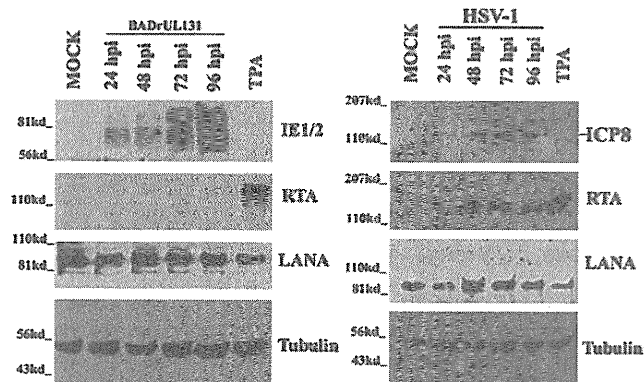

Fig. 2. KSHV reactivation in BCBL-1 cells by HCMV, HSV-1, and TPA. BCBL-1 cells were infected with vDW215-BADrUL131 (left) or HSV-1 (right) for the time indicated, and the cells were treated with TPA for 48 h and were collected and lysed in Lamile buffer; cellular proteins and viral proteins were detected with Western blot assay.

Several recent studies on the pathogenesis of KSHV have shown that a number of viruses (including HHV-6, HSV-1, HIV and HCMV) can induce KSHV lytic infection.[14,23,30,34] HCMV has been the focus of our interest not only because it can infect the same type of cells as KSHV – as has been shown by many laboratories – but also because it infects a large population (about 50–90%). An observation by Vieira et al. that HCMV can reactivate KSHV from latency might explain the virus–virus interactions.[30]

It is important to study KSHV latency disruption by HCMV in B lymphocytes because HCMV and KSHV can be detected in blood samples from the same patient, which suggests a co-infection. We infected BCBL-1 cells with vDW215-BADrUL131 HCMV at an MOI of 5 for different times as indicated in Fig. 2A. TPA-treated BCBL-1 was used as positive reactivation control. The whole-cell lysates were collected at the times indicated; Western blot was performed to demonstrate HCMV and KSHV proteins. An increase in IE1/2 production is evidence of active HCMV infection in BCBL-1 cells; faint bands above the major bands indicate small ubiquitin-like modifier (SUMO)-modified IE1/2. KSHV reactivation is indicated by the presence of RTA, a gene essential for KSVH reactivation; RTA can only be produced (and is the first gene expressed) after reactivation, as shown in TPA-treated BCBL-1 cells. The absence of RTA in HCMV-infected BCBL-1, then, suggests that HCMV failed to reactivate KSHV. Latency-associated nuclear antigen (LANA), a protein produced during KSHV latency on BCBL-1 cells and cellular protein (tubulin), was used as a sample loading control. As a control, we infected BCBL-1 cells with a wild-type strain of HSV-1, as shown in Fig. 2B, and ICP8 was used to indicate HSV-1 infection. We demonstrated that HSV-1 infection in BCBL-1 cells resulted in the

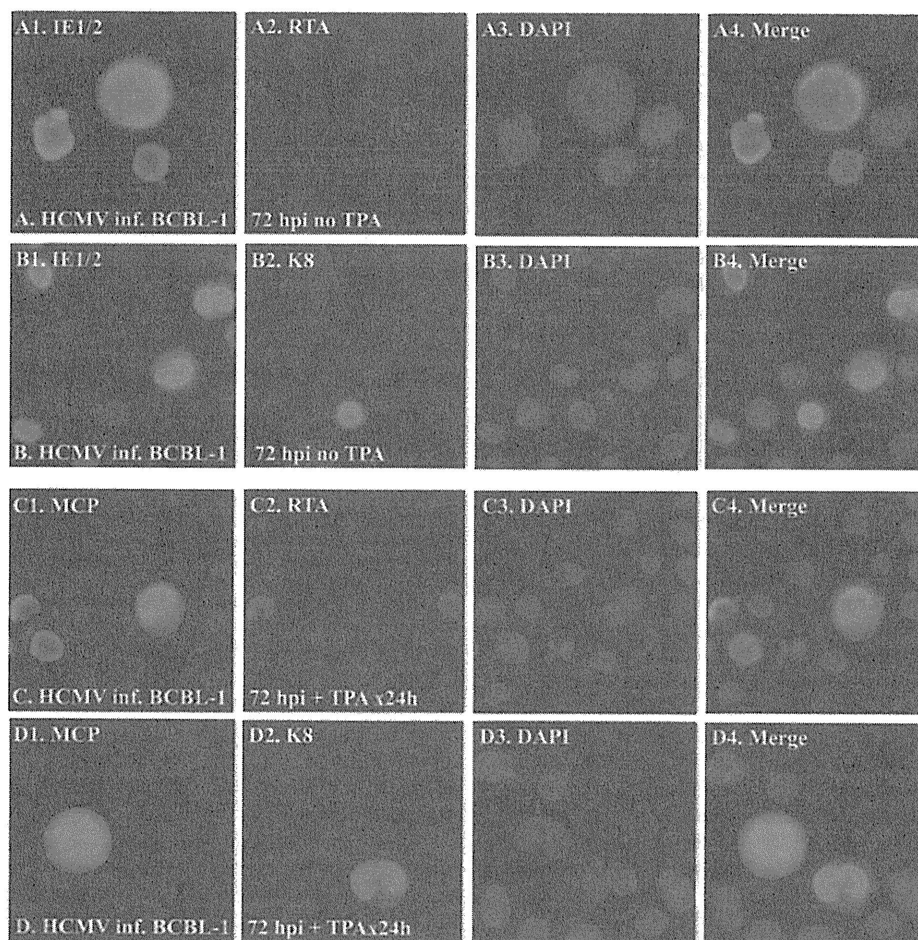

Fig. 3. Immunofluorescence to detect HCMV infection in BCBL-1 cells. BCBL-1 cells were infected with vDW215-BADrUL131, untreated (A and B) or treated (C and D) with TPA, and were washed with PBS and fixed with 1% paraformaldehyde and cytospun to slides for immunofluorescence assay using different antibioses, as indicated, to detect viral proteins: IE1/2 (A1 and B1) and MCP (C1 and D1) of HCMV in green; RTA (A2 and C2) and K8 (B2 and D2) of KSHV in red. Total cells were shown with DAPI blue (A3 to D3). Three different colors were merged and shown in A4–D4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
KSHV reactivation rate in HCMV infected cells versus non-infected cells. Relationship between HCMV infection and KSHV reactivation. Forty-eight hours after infection of BCBL-1 cells with vDW215-BADrUL131 at an MOI of 5, we added TPA in the cell culture for 24 h. The cells were cytospun to slides for detection with immunofluorescence with anti-HCMV IE1/2 and KSHV RTA antibody. Cells were chosen at random and counted.

HCMV infection state	Total number of cells counted	Number of RTA positive cells	Reactivation rate (%)
IE1/2 positive	300	35	11.7
IE1/2 negative	500	59	11.8

Student' t-test: $P > 0.05$.

reactivation of KSHV as indicated by the presence of RTA, which is consistent with the report from another group.[24] A question about the clinical significance of the observation of interaction of HSV-1 with KSHV still remains. An animal model might be needed to determine whether HSV-1 can reactivate KSHV.

Furthermore, we visualised the HCMV infection in BCBL-1 cells by immunofluorescence. First, when the BCBL-1 cells were infected with vDW215-BADrUL131 at an MOI of 5 for 72 h, the positive infection was shown by the production of IE1 (Fig. 3A1 and B1) and major capsid protein (MCP) in the nuclei (C1 and D1) in green. KSHV protein production was shown in red using anti-RTA polyclonal antibody (Fig. 3A2 and C2) and anti-K8 monoclonal antibody (Fig. 3B2 and D2), which signal the reactivation of KSHV because they are both lytic-stage proteins. The total cells in the microscope field were shown by 4′,6-diamidino-2-phenylindole (DAPI) staining in blue (A3–D3). The immunofluorescence studies showed that neither RTA nor K8 was detected in most of the HCMV positively infected cells. Three single colours were merged (A4–D4) to be able to visualise the relationship between HCMV infection and KSHV reactivation. It has been noticed that there is a positive cell in Fig. 3B2 (positive K8, in red) in which HCMV IE1/2 is much less than other cells in the same microscope field (Fig. 3B1). In BCBL-1 cell cultures that have not been stimulated, KSHV was reactivated (called leaking) in less than 1% of the cells; Fig. 3B1 depicts one of them. This immunofluorescence study further demonstrated that HCMV infection failed to reactivate KSHV in BCBL-1 cells.

We next queried whether HCMV could affect KSHV reactivation by TPA in BCBL-1 cell. After we infected BCBL-1 cells with vDW215-BADrUL131 at an MOI of 5 for 48 h, we added TPA in the cell culture for 24 h. The cells were cytospun to slides for detection with immunofluorescence with anti-HCMV MCP antibody (Fig. 3C and D) or with anti-IE1/2 (Table 1) and KSHV RTA or K8 antibody. As shown in Fig. 3C, HCMV infected cells (MCP positive: C1, left) can be still reactivated by TPA (RTA in red, C2). We also randomly counted the cells in two groups: HCMV infected (shown by IE1/2 in green) and non-infected to see whether there is any difference between the KSHV reactivation rates. As shown in Table 1, we counted 300 HCMV-infected cells in which 35 cells were reactivated (as marked with RTA staining). In 500 non-infected cells, 59 cells were reactivated. No significant difference was found between the two groups using Student's t-test.

3. Discussion

Human herpesviruses mostly cause latent infection in immune-competent populations and results in disease when reactivated by many different environmental conditions.[28] Several herpesviruses might infect the same person because many of them have high infection incidence. KSHV infection in the general population is not as high as other herpesviruses, but it is the first important pathogen causing cancer in AIDS patients. HCMV infects most of the population and can be occasionally reactivated.[18] Mixed infection of KSHV and HCMV intrigued us to investigate the interaction of the two viruses. The finding that HSV-1 infection can switch KSHV infection to the lytic stage is further demonstrated in this study; however, interactions between HCMV, KSHV and HIV might be of more importance because those viruses could infect the same kind of cells.

The facts that HCMV IE1 and IE2 can interact with HDAC and inhibit HDAC activities and that HDAC inhibitors can reactivate KSHV in cell culture suggest that HCMV infection might be able to reactivate KSHV infection. As a matter of fact, in the human fibroblast cell system, it has been reported recently that HCMV UL112/113 molecule can lead KSHV infection from latency to lytic stage in human fibroblast cells.[33] It is necessary to know whether HCMV could also reactivate KSHV in another cell line. We infected BCBL-1 harbouring KSHV genome with vDW215-BADrUL131 HCMV and detected the state of KSHV; it is clear that HCMV infection failed to reactivate KSHV in BCBL-1 cells. We also found that KSHV reactivation by TPA was not affected by HCMV infection. We also found that the two viruses can exist in the same cells and continue to express their own gene products. The differences in the results between our experiments and the other groups' regarding the effect of HCMV infection on KSHV reactivation imply a different mechanism of reactivation of KSHV in different types of cells.

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References

1. Akter P, Cunningham C, McSharry BP, Dolan A, Addison C, Dargan DJ, et al. Two novel spliced genes in human cytomegalovirus. *J Gen Virol* 2003;**84**:1117–22.
2. Ambroziak JA, Blackbourn DJ, Herndier BG, Glogau RG, Gullett JH, McDonald AR, et al. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. *Science* 1995;**268**:582–3.
3. Atula T, Grenman R, Klemi P, Syrjanen S. Human papillomavirus, Epstein-Barr virus, human herpesvirus 8 and human cytomegalovirus involvement in salivary gland tumours. *Oral Oncol* 1998;**34**:391–5.
4. Bechtel JT, Winant RC, Ganem D. Host and viral proteins in the virion of Kaposi's sarcoma-associated herpesvirus. *J Virol* 2005;**79**:4952–64.
5. Boshoff C, Whitby D, Talbot S, Weiss RA. Etiology of AIDS-related Kaposi's sarcoma and lymphoma. *Oral Dis* 1997;**3 Suppl. 1**:S129–32.
6. Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N Engl J Med* 1995;**332**:1186–91.
7. Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994;**266**:1865–9.
8. Deng H, Liang Y, Sun R. Regulation of KSHV lytic gene expression. *Curr Top Microbiol Immunol* 2007;**312**:157–83.
9. Fujimuro M, Nakaso K, Nakashima K, Sadanari H, Hisanori I, Teishikata Y, et al. Multiplex PCR-based DNA array for simultaneous detection of three human herpesviruses, EVB, CMV and KSHV. *Exp Mol Pathol* 2006;**80**:124–31.
10. Hahn G, Revello MG, Patrone M, Percivalle E, Campanini G, Sarasini A, et al. Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. *J Virol* 2004;**78**:10023–33.
11. Hassan-Walker AF, Mattes FM, Griffiths PD, Emery VC. Quantity of cytomegalovirus DNA in different leukocyte populations during active infection in vivo and the presence of gB and UL18 transcripts. *J Med Virol* 2001;**64**:283–9.
12. Kozireva S, Nemceva G, Danilane I, Pavlova O, Blomberg J, Murovska M. Prevalence of blood-borne viral infections (cytomegalovirus, human herpesvirus-6, human herpesvirus-7, human herpesvirus-8, human T-cell lymphotropic

virus-I/II, human retrovirus-5) among blood donors in Latvia. *Ann Hematol* 2001;**80**:669–73.
13. Krishnan HH, Naranatt PP, Smith MS, Zeng L, Bloomer C, Chandran B. Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. *J Virol* 2004;**78**: 3601–20.
14. Lu C, Zeng Y, Huang Z, Huang L, Qian C, Tang G, et al. Human herpesvirus 6 activates lytic cycle replication of Kaposi's sarcoma-associated herpesvirus. *Am J Pathol* 2005;**166**:173–83.
15. Lucht E, Brytting M, Bjerregaard L, Julander I, Linde A. Shedding of cytomegalovirus and herpesviruses 6, 7, and 8 in saliva of human immunodeficiency virus type 1-infected patients and healthy controls. *Clin Infect Dis* 1998;**27**:137–41.
16. Meer S, Altini M. Cytomegalovirus co-infection in AIDS-associated oral Kaposi's sarcoma. *Adv Dent Res* 2006;**19**:96–8.
17. Miller G, Heston L, Grogan E, Gradoville L, Rigsby M, Sun R, et al. 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* 1997;**71**:314–24.
18. Mocarski E. *Cytomegalovirus and their replication*. Philadelphia: Lippincott, Williams and Wilkins; 2001.
19. Moore PS, Boshoff C, Weiss RA, Chang Y. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* 1996;**274**:1739–44.
20. Murphy E, Yu D, Grimwood J, Schmutz J, Dickson M, Jarvis MA, et al. Coding potential of laboratory and clinical strains of human cytomegalovirus. *Proc Natl Acad Sci U S A* 2003;**100**:14976–81.
21. Nevels M, Paulus C, Shenk T. Human cytomegalovirus immediate-early 1 protein facilitates viral replication by antagonizing histone deacetylation. *Proc Natl Acad Sci U S A* 2004;**101**:17234–9.
22. Nishiwaki M, Fujimuro M, Teishikata Y, Inoue H, Sasajima H, Nakaso K, et al. Epidemiology of Epstein-Barr virus, cytomegalovirus, and Kaposi's sarcoma-associated herpesvirus infections in peripheral blood leukocytes revealed by a multiplex PCR assay. *J Med Virol* 2006;**78**:1635–42.
23. Qin D, Zeng Y, Qian C, Huang Z, Lv Z, Cheng L, et al. Induction of lytic cycle replication of Kaposi's sarcoma-associated herpesvirus by herpes simplex virus type 1: involvement of IL-10 and IL-4. *Cell Microbiol* 2007.
24. Qin D, Zeng Y, Qian C, Huang Z, Lv Z, Cheng L, et al. Induction of lytic cycle replication of Kaposi's sarcoma-associated herpesvirus by herpes simplex virus type 1: involvement of IL-10 and IL-4. *Cell Microbiol* 2008;**10**:713–28.
25. Soulier J, Grollet L, Oksenhendler E, Cacoub P, Cazals-Hatem D, Babinet P, et al. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 1995;**86**:1276–80.
26. Staskus KA, Zhong W, Gebhard K, Herndier B, Wang H, Renne R, et al. Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. *J Virol* 1997;**71**:715–9.
27. Tang Q, Maul GG. Mouse cytomegalovirus immediate-early protein 1 binds with host cell repressors to relieve suppressive effects on viral transcription and replication during lytic infection. *J Virol* 2003;**77**:1357–67.
28. Tarp B, Jensen-Fangel S, Dahl R, Obel N. Herpesvirus type 1-8 in BAL fluid from HIV-1-infected patients with suspected pneumonia and from healthy individuals. *Eur Respir J* 2001;**18**:146–50.
29. van der Kuyl AC, Polstra AM, van den Burg R, Jan Weverling G, Goudsmit J, Cornelissen M. Cytomegalovirus and human herpesvirus 8 DNA detection in peripheral blood monocytic cells of AIDS patients: correlations with the presence of Kaposi's sarcoma and CMV disease. *J Med Virol* 2005;**76**:541–6.
30. Vieira J, O'Hearn P, Kimball L, Chandran B, Corey L. Activation of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) lytic replication by human cytomegalovirus. *J Virol* 2001;**75**:1378–86.
31. Wang D, Shenk T. Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. *J Virol* 2005;**79**:10330–8.
32. Wang D, Shenk T. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. *Proc Natl Acad Sci U S A* 2005;**102**:18153–8.
33. Wells R, Stensland L, Vieira J. The HCMV UL112-113 locus can activate the full KSHV lytic replication cycle. *J Virol* 2009.
34. Zeng Y, Zhang X, Huang Z, Cheng L, Yao S, Qin D, et al. Intracellular Tat of human immunodeficiency virus type 1 activates lytic cycle replication of Kaposi's sarcoma-associated herpesvirus: role of JAK/STAT signaling. *J Virol* 2007;**81**:2401–17.

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Detection of Merkel Cell Polyomavirus in Merkel Cell Carcinoma and Kaposi's Sarcoma

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Merkel cell carcinoma is a rare malignancy that sometimes occurs in the skin of elderly people. Recently, a new human polyomavirus, Merkel cell polyomavirus (MCPyV) was identified in Merkel cell carcinoma. In the present study, MCPyV-DNA was detected in 6 of 11 (55%) cases of Merkel cell carcinoma by nested PCR and real-time PCR. Histologically, MCPyV-positive cases showed round and vesicular nuclei with a fine granular chromatin and small nucleoli, whereas MCPyV-negative cases showed polygonal nuclei with diffusely distributed chromatin. Real-time PCR analysis to detect the MCPyV gene revealed that viral copy numbers ranged 0.04–0.43 per cell in cases of Merkel cell carcinoma. MCPyV was also detected in 3 of 49 (6.1%) cases of Kaposi's sarcoma (KS), but not in 192 DNA samples of other diseases including 142 autopsy samples from 20 immunodeficient patients. The MCPyV copy number in KS was lower than that in Merkel cell carcinoma. PCR successfully amplified a full-length MCPyV genome from a case of KS. Sequence analysis revealed that the MCPyV isolated from KS had 98% homology to the previously reported MCPyV genomes. These data suggest that the prevalence of MCPyV is low in Japan, and is at least partly associated with the pathogenesis of Merkel cell carcinoma. ***J. Med. Virol. 81:1951–1958, 2009.***
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KEY WORDS: Merkel cell polyomavirus; Merkel cell carcinoma; real-time PCR; Kaposi's sarcoma

INTRODUCTION

Merkel cell carcinoma is a rare skin malignancy that originates in the Merkel cell, a neuroendocrine cell in the skin [Skelton et al., 1997; Pectasides et al., 2006; Lemos and Nghiem, 2007]. Merkel cell carcinoma is an aggressive skin cancer that commonly occurs on sun-exposed areas of elderly people. The incidence of Merkel cell carcinoma is high in Caucasians, but lower in other races [Agelli and Clegg, 2003]. Typical histology of Merkel cell carcinoma shows that the tumor consists of small, round cells with vesicular nuclei, fine chromatin, and minimal cytoplasm. Because these morphological features are similar to other small cell tumors such as small cell lung cancer and lymphoma, immunohistochemistry for cytokeratin 20 is often useful for differential diagnosis. Recurrence and metastasis in the regional lymph nodes are observed in up to 30% of cases [Agelli and Clegg, 2003]. Merkel cell carcinoma comprises two subtypes, "classic" and "variant," based on clinical manifestations and gene expression profiles [Van Gele et al., 2004; Fernandez-Figueras et al., 2007]. Gene expression profiling studies have also demonstrated that metastasis of Merkel cell carcinoma is correlated with the over-expression of some metastasis-associated proteins such as matrix metalloproteinases [Fernandez-Figueras et al., 2007].

A new human polyomavirus, Merkel cell polyomavirus (MCPyV), was identified recently in samples of Merkel cell carcinoma by digital transcriptome subtraction [Feng et al., 2008]. MCPyV is a 5.4-kbp long, double-stranded DNA virus and has high

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homology to murine, African green monkey, and hamster polyomaviruses. The MCPyV genome encodes a large T antigen, which contains some conserved domains that were shown previously to play roles in cell transformation. In an earlier report, MCPyV was detected in 8 out of 10 cases of Merkel cell carcinoma (80%) by PCR, and clonal integration into the human genome was observed in 6 of the 8 cases with Merkel cell carcinoma [Feng et al., 2008]. Several recent reports have demonstrated the presence of MCPyV in Merkel cell carcinoma cases in the United States, Europe, and Australia, but is rare in other samples such as melanoma, skin cancer, other cancers, and controls [Foulongne et al., 2008; Giraud et al., 2008; Kassem et al., 2008; Becker et al., 2009; Bialasiewicz et al., 2009; Bluemn et al., 2009; Duncavage et al., 2009; Garneski et al., 2009; Goh et al., 2009; Ridd et al., 2009; Sharp et al., 2009]. A study using frozen samples of Merkel cell carcinoma demonstrated that all Merkel cell carcinoma samples were positive for MCPyV-DNA [Sastre-Garau et al., 2009]. These data strongly suggest that MCPyV infection is associated with the pathogenesis of Merkel cell carcinoma [zur Hausen, 2008]. On the other hand, studies using paraffin-embedded tissues suggest the presence of some MCPyV-negative cases of Merkel cell carcinoma [Foulongne et al., 2008; Kassem et al., 2008; Becker et al., 2009; Duncavage et al., 2009; Garneski et al., 2009]. In addition, MCPyV is less frequently present in Merkel cell carcinoma in Australian cases than in North American cases, suggesting geographic differences of MCPyV distribution [Garneski et al., 2009]. Although there are no accurate statistical data, Merkel cell carcinoma seems to be rare in Japan. In this study, the presence of MCPyV was investigated in Japanese cases of Merkel cell carcinoma and other diseases.

MATERIALS AND METHODS

Specimens

Studies using human tissue were performed with the approval of the Institutional Review Board of the National Institute of Infectious Diseases (Approval No. 149). Thirteen formalin-fixed paraffin-embedded tissue samples of Merkel cell carcinoma were collected from 11 patients (Table I). All of the samples were taken by biopsy or from tumor resection, and were enriched in tumor cells. The diagnosis of Merkel cell carcinoma was based on morphology and immunohistochemistry of cytokeratin 20. In addition to the Merkel cell carcinoma samples, 49 DNA samples of Kaposi's sarcoma (KS) and 50 DNA samples from tissues with various diseases were collected. A further 142 DNA samples were extracted from various organs of 20 autopsy cases with AIDS. All these human samples were archived as anonymous specimens held at the Department of Pathology, National Institute of Infectious Diseases. DNA extracted from 15 cell lines was also investigated.

TABLE I. MCPyV Infection in Merkel Cell Carcinoma Cases

Clinical information				Nested PCR for MCPyV						Real-time PCR		
Case	Sex	Age	Site	ST	LT (1017–1170)	LT (2057–2107)	VP1	VP2	VP3	MCPyV copy per 100 ng DNA	β-Actin copy per 100 ng DNA	Predicted MCPyV copy per cell
1	F	89	Cheek	−	−	−	−	−	−	0	17,505	0.000
2	F	104	Cheek	+	+	+	+	+	+	356	1,645	0.433
3	F	76	Forearm	+	+	−	−	−	−	247	12,980	0.038
4	F	50	Face	+	+	+	+	+	+	617	7,300	0.169
5	M	87	Face	−	−	−	−	−	−	0	2,053	0.000
6	M	90	Head	−	−	−	−	−	−	0	670	0.000
7	F	89	Face	−	−	−	−	−	−	0	11,940	0.000
8	F	95	Inguinal	−	−	−	−	−	−	0	39,865	0.000
8*	F	95	Abdomen	−	−	−	−	−	−	0	13,520	0.000
9	F	86	Face	+	+	+	+	+	+	691	18,960	0.073
9*	F	86	Face	+	+	−	+	+	+	78	2,796	0.056
10	M	78	Forearm	+	+	−	+	+	+	93	911	0.206
11	F	96	Cheek	+	+	−	+	−	−	50	423	0.119

Samples 8* and 9* are recurrent tumors of cases 8 and 9, respectively.

Preparation of DNA

DNA was extracted from formalin-fixed paraffin-embedded or fresh-frozen clinical materials. To isolate DNA from formalin-fixed paraffin-embedded biopsies, 5-μm-thick sections (n = 3–4) were placed into sterile eppendorf tubes, deparaffinized with xylene, digested with proteinase K, and processed for phenol/chloroform extraction with sodium acetate/ethanol precipitation. For fresh-frozen materials, DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's instructions.

Nested PCR

The small T (ST), large T (LT), and VP1–3 regions of MCPyV were amplified by nested PCR. The primer sequences are listed in Table II. The first round of amplification was performed with 100 ng of extracted DNA and high fidelity Taq DNA polymerase (Roche Diagnostics, Boehringer Mannheim, Germany) in a final volume of 25 μl. After an initial DNA denaturation for 2 min at 94°C, the samples were amplified for 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a final elongation phase of 7 min at 72°C. The second round of amplification was performed with 1 μl of the first round PCR product in a final volume of 25 μl under the following parameters: 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec for 25 cycles, followed by a final elongation phase of 7 min at 72°C. Five microliters of amplification products were loaded onto agarose gels, electrophoresed, stained with bromide and visualized under UV light. The β-globin gene was amplified as an internal control by single PCR [Katano et al., 2001].

DNA Sequencing

The PCR products from nested PCR were sequenced directly with an ABI sequencer 3730 (Applied Biosystems, Foster City, CA) using a dye terminator ready reaction kit (Applied Biosystems) according to the manufacturer's instructions.

Real-Time PCR

Copy numbers of MCPyV-DNA were determined by quantitative real-time TaqMan PCR using the Mx3005P real-time PCR system (Stratagene, La Jolla, CA), which amplified a segment within the LT (1017–1170) domain. The amount of human genomic DNA (β-actin gene) present in the DNA extracted from each specimen was also determined. Primers and probes for the MCPyV-LT gene were designed using Primer Express software (Applied Biosystems). To amplify MCPyV-LT, forward (Merkel PV LT Forward: 5′-TCTGGGTATGGGTCCTT-CTCA-3′) and reverse (Merkel PV LT Reverse: 5′-TGG-TGTTCGGGAGGTATATCG-3′) primers were used with a labeled probe 5′-(FAM)CGTCCCAGGCTTCAGACTC-CCAGTC(TAMRA)-3′. To amplify β-actin DNA, forward (5′-TGAGCGCGGCTACAGCTT-3′) and reverse (5′-TC-CTTAATGTCACGCACGATTT-3′) primers were used with a labeled probe 5′-(FAM)ACCACCACGGCCGAG-CGG(TAMRA)-3′ [Kuramochi et al., 2006]. The amplicon sizes of MCPyV-LT and β-actin were 77 bp (1053–1129 in GenBank EU375803) and 60 bp (655–714 in NM_001101), respectively. PCR amplification was performed in 20-μl reaction mixtures using QuantiTect probe PCR Master Mix (Qiagen), 0.3 μM of each primer, 0.3 μM of TaqMan probe, and 2 μl isolated

TABLE II. Primers Used for Nested PCR

Gene	Out/in	F/r	Primer name	5′–3′	Position*	Size (bp)
ST	Outer	Forward	MCV-ST-F515	CTGGGTGCATGCTTAAGCAAC	515–732	218
		Reverse	MCV-ST-R732	GCAGTAGTCAGTTTCTTCT		
	Inner	Forward	MCV-ST-F550	TGCGCTTGTATTAGCTGTAAGT	550–703	154
		Reverse	MCV-ST-R703	GCCACCAGTCAAAACTTTCCCA		
LT (1017–1170)	Outer	Forward	MCV-LT-F992	CTCCAATGCATCCAGGGGAG	992–1192	201
		Reverse	MCV-LT-R1192	TCTCTTCCTGAATTGGTGGT		
	Inner	Forward	MCV-LT-F1017	AGTGGAAGCTCACCACCCCACA	1017–1170	154
		Reverse	MCV-LT-R1170	CCTCTCTGCTACTGGATCCA		
LT (2057–2207)	Outer	Forward	MCV-LT-F2034	CCATTTCCTTGCCAAAAGTG	2034–2228	195
		Reverse	MCV-LT-R2228	CTTACATAGCATTTCTGTCC		
	Inner	Forward	MCV-LT-F2057	AAACAGATCTCGCCTCAAAC	2057–2207	150
		Reverse	MCV-LT-R2207	GGTCATTTCCAGCATCTCTA		
VP1	Outer	Forward	MCV-VP1-F4233	TGAATCCAAGAATGGGAGTT	4062–4252	191
		Reverse	MCV-VP1-R4062	CATCTGCAATGTGTCACAG		
	Inner	Forward	MCV-VP1-F4212	TCCCCTGATCTTCCTACT	4092–4229	138
		Reverse	MCV-VP1-R4092	ATTTAGCATTGGCAGAGAC		
VP2	Outer	Forward	MCV-VP2-F4490	CAATCTGGAGTTTGCTGCTG	4490–4692	203
		Reverse	MCV-VP2-R4692	CTGCATTCTGTGGGGCAAAAT		
	Inner	Forward	MCV-VP2-F4511	AGAGTTCCTCCTATATGTTC	4511–4661	151
		Reverse	MCV-VP2-R4661	TTTATCTCCTACCTCTAGGC		
VP3	Outer	Forward	MCV-VP3-F4890	CCAAAGAAGCCACTAATGAG	4890–5118	229
		Reverse	MCV-VP3-R5118	ATGGGGGGCATCATCACACTG		
	Inner	Forward	MCV-VP3-F4932	TGAACCCAAGTTGAGCTAAAGC	4932–5097	166
		Reverse	MCV-VP3-R5097	CTGGCCAATATTGGTGAAATTG		

*Position in GenBank EU375803.

DNA. The PCR conditions were: 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec and 60°C for 1 min. Quantitative results were obtained by generating standard curves for pCR2.1 plasmids (Invitrogen, Carlsbad, CA) containing each MCPyV-LT and the cellular target (β-actin gene) amplicon. Virus copy numbers per cell were calculated by dividing MCPyV-LT copy numbers by half of the β-actin copy numbers, because each cell contains two copies of DNA in two alleles [Asahi-Ozaki et al., 2006].

Histological and Immunohistochemical Analyses

Immunohistochemistry was performed to investigate the expression of cytokeratin 20, chromogranin and neuron-specific enolase (NSE) on paraffin-embedded tissues of Merkel cell carcinoma. Sections (4 μm thick) were deparaffinized by sequential immersion in xylene and ethanol, and rehydrated in distilled water. For antigen retrieval, the sections were autoclaved in 1 mM EDTA, 0.05 M Tris–HCl, pH 9.0, at 121°C for 10 min for cytokeratin 20 and chromogranin, or 0.01 M citrate buffer at 95°C for 10 min for NSE. Endogenous peroxidase activity was blocked by immersing the sections in methanol/0.6% H_2O_2 for 30 min at room temperature. Diluted anti-cytokeratin 20 (Dako, Copenhagen, Denmark), chromogranin (Dako), or NSE (Novocastra Laboratories, Newcastle, UK) antibodies were applied and the sections were incubated overnight at 4°C. After washing in PBS twice, a biotinyl anti-mouse IgG goat antibody and peroxidase-conjugated streptavidin were used as the secondary and tertiary antibodies, respectively. 3,3′-diaminobenzidine was used as a chromogen.

Cloning of a Full-Length MCPyV Genome

A full-length MCPyV genome was amplified by single PCR using KOD-FX DNA polymerase (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. A primer-set (primer set 1: MCV-*Bam*HI-1150F TCTGGATCCAGTAGCAGAGAGGAGACC and MCV-*Bam*HI-1150R CTCGGATCCAGAGGATGAGGTGGGTTC) was used to amplify the full-length MCV genome. Following the addition of dA to the PCR product, the PCR product was TA-cloned into pCR2.1 vector (Invitrogen). Primer set 2 (MCV-F133 CTTAGTGAGGTAGCTCATTTGCTCCTCTGCTGTT and MCV-R5325 AACTTTTATTGCTGCAGGGTTTCTGGCATTGACTC) was used to amplify almost the full-length of the MCPyV genome except for the origin region. A 1,188-bp-fragment containing the origin region was amplified using another primer set (primer set 3: MCV-VP3-F4932 TGAACCCAAGTTGAGCTAAAGC and MCV-ST-R732 GCAGTAGTCAGTTTCTTCT).

RESULTS

PCR Detection of MCPyV in Merkel Cell Carcinoma

To determine whether MCPyV is present in Japanese cases of Merkel cell carcinoma, PCR analysis was first performed on DNA samples extracted from 11 cases of Merkel cell carcinoma using primers specific for the MCPyV-LT and VP1 regions, as published previously [Feng et al., 2008]. Although the β-globin gene was amplified in all DNA samples, fragments of MCPyV were not detected using these primers (data not shown). Thus, nested PCR, which can detect gene fragments <250 bp in length, was performed for six MCPyV genes (Table II). Nested PCR revealed that 6 of the 11 Merkel cell carcinoma cases (54.5%) were positive for MCPyV ST and LT (1017–1170) fragments (Fig. 1 and Table I). Interestingly, LT (2057–2107) and VP2-3 were detected in cases 2, 4, and 9, but not in cases 3, 10, or 11. LT (2057–2107) was not detected in the recurrent samples of case 9. Sequencing analysis revealed that all these PCR products were fragments of MCPyV, and an A to G mutation was detected at the position 4589 of GenBank EU375803 in the VP2 fragments of cases 2, 4, 9, and 10 with Merkel cell carcinoma. In addition, two mutations, G4996C and A5032T, were detected in the VP3 fragments of cases 2, 4, 9, and 10, which were the same sequences as the MCC339 strain (GenBank EU375804). Cases 2, 4, and 9 showed doublet bands of 191 and 138 bp in the VP1 PCR. Sequencing analysis revealed that these two bands corresponded to PCR products of primary and secondary amplification. None of the primer sets for nested PCR amplified DNA products in tissues extracted from JCV-positive progressive multifocal leukoencephalopathy (PML) or BKV-positive nephritis samples (Fig. 1). These data indicate the presence of MCPyV in six cases of Merkel cell carcinoma.

Quantitative Analysis of MCPyV in Merkel Cell Carcinoma

To determine the virus copy numbers in DNA samples, a real-time TaqMan PCR to detect the MCPyV gene was established. Based on the results of nested

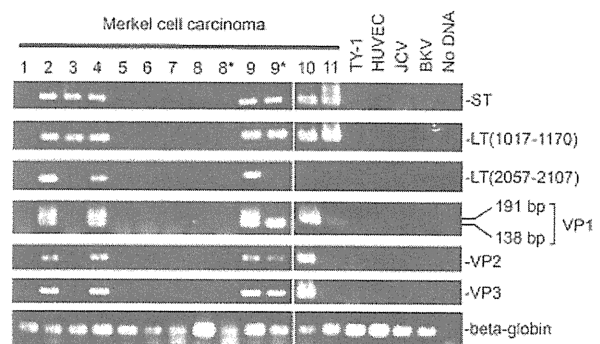

Fig. 1. Detection of MCPyV in Merkel cell carcinoma cases by nested PCR. Fragments of MCPyV were amplified with nested PCR in Merkel cell carcinoma cases. Thirteen samples of Merkel cell carcinoma were used. Sample nos. 8* and 9* are recurrent tumors of cases 8 and 9, respectively. TY-1, human herpesvirus-8-positive B cell line; HUVEC, human umbilical vascular endothelial cells; JCV, JCV-positive progressive multifocal leukoencephalopathy sample; BKV, BKV-positive nephritis sample. The two bands at 191 and 138 bp represent the PCR products of primary and secondary amplification, respectively. The lower panel shows single PCR for the β-globin gene.

PCR, the LT (1017–1170) region of MCPyV was selected as a target gene for real-time PCR. Amplification plots and standard curves revealed a linear relationship between copy numbers from 10 to 10^8 copies and cycle threshold (C_t), indicating that the dynamic range of the real-time PCRs was between 10 and 10^8 copies (data not shown). The MCPyV-LT genome assay uniformly detected 10 copies of pCR2.1-MCPyV-LT plasmid. PCR amplification of β-actin also uniformly detected 10 copies of the β-actin genome (data not shown). The specificity of the assay for MCPyV-LT was confirmed using a panel of DNAs from other polyomavirus (JCV, BKV, and SV40), human papillomavirus, herpes viruses (herpes simplex virus-1 and -2, varicella zoster virus, EBV, human cytomegalovirus, and HHV-6, -7, and -8), and cellular DNA from human cell lines (TY-1 and Raji, data not shown). Real-time PCR revealed that all six cases (seven samples) positive for MCPyV by nested PCR had 50–691 copies per 100 ng DNA (Table I). Compared with the results of cellular DNA (β-actin), the copy numbers in MCPyV-positive samples were estimated to in the range of 0.04–0.43 copies per cell (Table I).

Morphological Features of MCPyV-Positive Merkel Cell Carcinoma

Histological analysis of Merkel cell carcinoma cases revealed some morphological differences between MCPyV-positive and -negative Merkel cell carcinoma cases. All seven MCPyV-positive samples (cases 2, 3, 4, 9, 9*, 10, and 11) had common features such as round and vesicular nuclei with fine granular chromatin and small nucleoli. However, MCPyV-negative samples showed various morphologies. Some of the MCPyV-negative cases showed some similarities in morphology with MCPyV-positive samples; however, most of the MCPyV-negative samples had polygonal nuclei with light cytoplasm (Fig. 2). Chromatin was diffusely distributed in the nuclei, and the nucleoli were unclear in MCPyV-negative samples. MCPyV-positive cases also showed diffuse infiltration into the skin, whereas some of the MCPyV-negative cases showed duct differentiation. There were no significant differences in cytokeratin 20, NSE, or chromogranin expression between the MCPyV-positive and -negative Merkel cell carcinoma samples (Fig. 2 and data not shown). These morphological differences suggest differences in pathogenesis between MCPyV-positive and -negative Merkel cell carcinoma.

Prevalence of MCPyV in Various Diseases and Cell Lines

To clarify the prevalence of MCPyV, real-time PCR for MCPyV-LT was performed on DNAs extracted from various tissue samples (Table III). MCPyV was detected in three out of 49 (6.1%) cases of KS, but not in other diseases such as AIDS-related lymphoma, fulminant hepatitis, encephalitis, primary pulmonary hypertension, or necrotizing lymphadenitis. All 142 autopsy samples taken from various organs of 20 immunodeficient patients were negative for MCPyV with real-time PCR. In addition, all DNA samples extracted from 15 cell lines (seven B cell lines, TY-1, BCBL-1, Raji, Namalwa, Bjab, KS1, and OS1; three T cell lines, MT4, Molt4, and Jurkat; two monocyte cell lines, HL60, and THP1; one KS cell line, 22-KS; one endothelial cell line, HUVEC; one Marmoset cell line, B95-8) were negative for MCPyV (data not shown). These data indicate the low prevalence of MCPyV in Japan, and suggest that some cases of Merkel cell carcinoma and KS are associated with MCPyV infection.

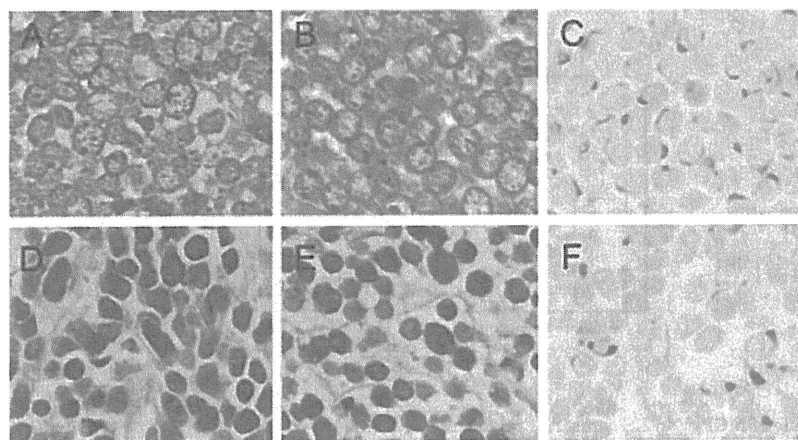

Fig. 2. Histology of MCPyV-positive and -negative Merkel cell carcinoma. **A–C**: MCPyV-positive Merkel cell carcinoma. HE staining of cases 2 (A) and 9 (B) shows round and vesicular nuclei with fine granular chromatin and small nucleoli. C: Cytokeratin 20 expression of case 2. **D–F**: MCPyV-negative Merkel cell carcinoma. HE staining of cases 5 (D) and 6 (E) shows dark and polymorphic nuclei with light cytoplasm. Chromatin in the polymorphic nuclei is diffuse, and the nucleoli are unclear. F: Cytokeratin 20 expression in case 6 is similar to the MCPyV-positive cases.

TABLE III. MCPyV Infection in Various Diseases

Sample	Positive/all	Percentage
Merkel cell carcinoma	6 (0)/11 (0)	54.5
KS	3 (1)/49 (16)	6.1
AIDS-related lymphoma	0 (0)/11 (11)	0
Fulminant hepatitis	0 (0)/9 (9)	0
Encephalitis	0 (0)/12 (12)	0
Including PML (JCV+)	0 (0)/4 (4)	0
BKV-positive nephritis	0 (0)/1 (1)	0
Primary effusion lymphoma	0 (0)/4 (4)	0
PPH	0 (0)/10 (0)	0
Necrotizing lymphadenitis	0 (0)/2 (2)	0
FDC sarcoma	0 (0)/1 (1)	0
AIDS autopsy		
Brain	0 (0)/15 (15)	0
Tongue	0 (0)/5 (5)	0
Submandibular gland	0 (0)/5 (5)	0
Lung	0 (0)/15 (15)	0
Lymph node	0 (0)/12 (12)	0
Heart	0 (0)/9 (9)	0
GI tract	0 (0)/13 (13)	0
Liver	0 (0)/16 (16)	0
Spleen	0 (0)/19 (19)	0
Pancreas	0 (0)/12 (12)	0
Kidney	0 (0)/14 (14)	0
Adrenal gland	0 (0)/7 (7)	0

KS, Kaposi's sarcoma; PML, progressive multifocal leukoencephalopathy; PPH, primary pulmonary hypertension; FDC, follicular dendritic cell; GI, gastrointestinal.
The number of frozen samples is shown in parentheses.

MCPyV Infection in KS Samples and Cloning of a Full-Length MCPyV Genome From a KS Case

Nested PCR was performed to confirm MCPyV infection in KS samples positive for MCPyV on real-time PCR. MCPyV fragments were detected in three KS cases using nested PCR (KS cases 3, 14, and 36 in Fig. 3A). However, nested PCR failed to detect VP1 and VP3 in KS cases 3 and 14, respectively. Sequencing analysis revealed that these products had sequences identical to those of Merkel cell carcinoma cases in the present study. The MCPyV-LT/β-actin copy numbers in these three KS samples were $1.3 \times 10^1/2.0 \times 10^4$, $3.3 \times 10^1/2.4 \times 10^5$, and $3.3 \times 10^1/1.8 \times 10^6$ per 100 ng DNA, respectively, suggesting low copy numbers (3.6×10^{-5}–1.2×10^{-3} copies per cell) compared with the Merkel cell carcinoma cases (Fig. 3B). Two (cases 14 and 36) of the MCPyV-positive cases with KS were AIDS-associated KS; however, the other case (case 3) was an HIV-negative patient. Because one MCPyV-positive KS case (case 36) was a frozen tissue sample, that sample was used to clone the full genome of MCPyV. PCR successfully amplified the full-length MCPyV genome with a primer set localized using the *Bam*HI site (1152 in GenBank EU375803) (primer set 1: MCV-*Bam*HI-1150F and MCV-*Bam*HI-1150R). PCR was also able to amplify almost the full-length of the MCPyV genome using a primer set localized to the origin region of MCPyV (primer set 2 MCV-F133 and MCV-R5325), and a 1,188-bp-fragment containing the origin region (primer set 3 MCV-VP3-F4932 and MCV-ST-R732), suggesting the presence of a MCPyV episome. The PCR product of full-length MCPyV genome was TA-cloned into pCR2.1 and two clones were sequenced. Sequence analysis revealed that the length of MCPyV genome was 5,418 bp. The two clones had the same sequence. This strain of MCPyV was designated as MCV-TKS (MCPyV-Tokyo Kaposi sarcoma) and registered in the GenBank (accession No. FJ464337). The sequence of MCV-TKS had 98% homology to MCC350 (GenBank EU375803). The motifs of LXXLL in CR1, HPDKGG in DnaJ, and LXCXE in the Rb binding site were conserved in the LT gene of MCV-TKS. The sequence of LT had a stop codon at 1,498, suggesting that MCV-TKS would produce a truncated form of LT [Shuda et al., 2008].

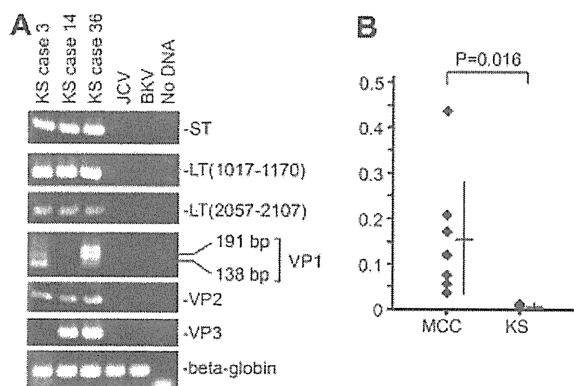

Fig. 3. Detection and cloning of MCPyV in KS cases. **A**: Fragments of MCPyV were amplified with nested PCR in three KS cases. JCV, JCV-positive PML sample; BKV, BKV-positive nephritis sample. **B**: Comparison of MCPyV copy numbers between Merkel cell carcinoma (MCC) and Kaposi's sarcoma (KS) samples with real-time PCR. The *y*-axis shows copy numbers per cell. The horizontal and vertical lines indicate means and standard deviation, respectively. The *P*-value was calculated with the Mann–Whitney test.

DISCUSSION

In the present study, the presence of MCPyV was demonstrated in Japanese cases of Merkel cell carcinoma. This indicates that MCPyV is distributed not only in the US, Europe, and Australia, but also worldwide. The rate of MCPyV in Japanese Merkel cell carcinoma cases (55%) was lower than that of the United States and European cases (69–100%), but higher than in Australia (24%) [Feng et al., 2008; Foulongne et al., 2008; Kassem et al., 2008; Becker et al., 2009; Duncavage et al., 2009; Garneski et al., 2009; Sastre-Garau et al., 2009]. In addition to that of Merkel cell carcinoma, the rate of MCPyV in KS (6%) was also lower than in the United States (16%) [Feng et al., 2008]. A US group detected MCPyV in some control tissues obtained from the gastrointestinal tract with PCR-Southern blot hybridization [Feng et al., 2008]. However, in the present study, all of the samples, except for Merkel cell carcinoma and KS samples, were negative for MCPyV. Taken together, these data suggest a lower infection rate of MCPyV in the Japanese population than in the US. Because Merkel cell carcinoma occurs more

frequently in Caucasians than in other races [Agelli and Clegg, 2003], it is possible that the distribution of MCPyV varies among races.

Nested PCR failed to detect VP1–3 and LT (2057–2107) in some MCPyV-positive cases of Merkel cell carcinoma including a recurrent case of MCPyV-positive tumor (Fig. 1). VP1 or VP3 was not detected in two MCPyV-positive cases of KS (Fig. 3A). There are some possible interpretations of these results. Recent reports demonstrated mutations in the LT gene and a unique deletion in the VP1 gene [Kassem et al., 2008; Shuda et al., 2008]. Thus, some mutations or deletions might exist in the primer regions of VP1–3 and LT of MCPyV, as in other polyomavirus such as JCV, BKV, and human papillomavirus (HPV).

Morphological differences between MCPyV-positive and -negative cases were found in the present study. MCPyV-positive cases can be categorized into one group with a typical morphology, whereas the MCPyV-negative cases had a mixed morphology. These morphological differences imply direct tumorigenesis by MCPyV infection in the MCPyV-positive cases and more complicated mechanisms in the MCPyV-negative cases. No rosette formation or ductal differentiation was observed in the MCPyV-positive cases, suggesting that MCPyV infection might be associated with differentiation of Merkel cells. A recent gene expression analysis using microarrays revealed that Merkel cell carcinoma might comprise two subtypes, namely the classic and variant types [Van Gele et al., 2004]. Although it is presumed that MCPyV infection may be associated with such subtypes, more cases of Merkel cell carcinoma are required to clarify the morphological features and presence of MCPyV infection. In addition, a recent immunohistochemical study demonstrated the expression of MCPyV-LT protein in tumor cells in Merkel cell carcinoma with a high number of MCPyV copies per cell, suggesting that MCPyV causes a subset of Merkel cell carcinoma [Shuda et al., 2009]. The morphological differences between MCPyV-positive and -negative cases support the presence of a MCPyV-negative subset of Merkel cell carcinoma.

The data for virus copy number provide valuable insights into the pathogenesis of Merkel cell carcinoma. In the present study, nested PCR and real-time PCR, but not PCR-Southern blot hybridization, was performed to detect MCPyV in clinical samples. Because DNA is always cleaved into small fragments in formalin-fixed tissues, it is difficult to amplify long DNA fragments. Our nested PCR and real-time PCR targeted fragments of MCPyV of <250 bp. The results of nested PCR for the LT gene and real-time PCR on clinical samples were well-correlated with each other in the present study. Because other studies have demonstrated that MCPyV-DNA is integrated into genomic DNA, at least one or two copies of MCPyV per cells are expected [Feng et al., 2008; Sastre-Garau et al., 2009]. On the other hand, it was demonstrated that Merkel cell carcinoma cases with a low copy number of MCPyV did not express the LT antigen [Shuda et al., 2009]. Because Merkel cell carcinoma tissue samples contained various proportions of tumor cells and normal cells, it is difficult to determine the accurate virus copy number per cell [Asahi-Ozaki et al., 2006]. The Merkel cell carcinoma tissue samples in the present study were rich in tumor cells and contained relatively few normal cells. Thus, the copy numbers of MCPyV in Merkel cell carcinoma (0.04–0.43 per cell) in the present study suggest the presence of Merkel cell carcinoma in cases with a low copy number of MCPyV. It was not possible to examine the clonal integration of MCPyV in DNA samples extracted from formalin-fixed and paraffin-embedded tissues. Future studies on frozen tissue samples will reveal the association between pathogenesis and MCPyV integration.

Here, MCPyV was detected in three cases of KS and cloning a full-length MCPyV genome. A previous report also demonstrated the presence of MCPyV in some KS cases without HIV infection [Feng et al., 2008]. Therefore, there might be some association between MCPyV infection and KS pathogenesis. There was no common feature between the three MCPyV-positive KS cases, including HIV-status. Human herpesvirus-8 was identified in all KS cases examined (data not shown). However, none of the Merkel cell carcinoma cases used in the present study were positive for human herpesvirus-8, as determined by PCR (data not shown). The results using AIDS autopsy samples also suggest that HIV status was not correlated with MCPyV infection. The low MCPyV copy number and the presence of episomal MCPyV in the KS samples suggest an indirect association between MCPyV and KS pathogenesis. MCV-TKS identified in the present study is the first isolate of MCPyV derived from a KS sample. The sequence data for MCV-TKS suggested that the LT gene would produce truncated LT, which is associated with the pathogenesis of Merkel cell carcinoma [Shuda et al., 2008]. However, to determine whether MCPyV infection is a risk factor for KS, the MCPyV status in Merkel cell in KS patients should be investigated.

In conclusion, MCPyV was detected in Japanese cases with Merkel cell carcinoma and KS. Although MCPyV infection appears to be rare in the Japanese population, this study provides evidence that MCPyV is spread worldwide, and that MCPyV is associated with pathogenesis in some Merkel cell carcinoma cases.

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REFERENCES

Agelli M, Clegg LX. 2003. Epidemiology of primary Merkel cell carcinoma in the United States. J Am Acad Dermatol 49:832–841.

Asahi-Ozaki Y, Sato Y, Kanno T, Sata T, Katano H. 2006. Quantitative analysis of Kaposi sarcoma-associated herpesvirus (KSHV) in KSHV-associated diseases. J Infect Dis 193:773–782.

Becker JC, Houben R, Ugurel S, Trefzer U, Pfohler C, Schrama D. 2009. MC polyomavirus is frequently present in Merkel cell carcinoma of European patients. J Invest Dermatol 129:248–250.

Bialasiewicz S, Lambert SB, Whiley DM, Nissen MD, Sloots TP. 2009. Merkel cell polyomavirus DNA in respiratory specimens from children and adults. Emerg Infect Dis 15:492–494.

Bluemn EG, Paulson KG, Higgins EE, Sun Y, Nghiem P, Nelson PS. 2009. Merkel cell polyomavirus is not detected in prostate cancers, surrounding stroma, or benign prostate controls. J Clin Virol 44: 164–166.

Duncavage EJ, Zehnbauer BA, Pfeifer JD. 2009. Prevalence of Merkel cell polyomavirus in Merkel cell carcinoma. Mod Pathol 22:516–521.

Feng H, Shuda M, Chang Y, Moore PS. 2008. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 319:1096–1100.

Fernandez-Figueras MT, Puig L, Musulen E, Gilaberte M, Lerma E, Serrano S, Ferrandiz C, Ariza A. 2007. Expression profiles associated with aggressive behavior in Merkel cell carcinoma. Mod Pathol 20:90–101.

Foulongne V, Kluger N, Dereure O, Brieu N, Guillot B, Segondy M. 2008. Merkel cell polyomavirus and Merkel cell carcinoma, France. Emerg Infect Dis 14:1491–1493.

Garneski KM, Warcola AH, Feng Q, Kiviat NB, Leonard JH, Nghiem P. 2009. Merkel cell polyomavirus is more frequently present in North American than Australian Merkel cell carcinoma tumors. J Invest Dermatol 129:246–248.

Giraud G, Ramqvist T, Ragnarsson-Olding B, Dalianis T. 2008. DNA from BK virus and JC virus and from KI, WU, and MC polyomaviruses as well as from simian virus 40 is not detected in non-UV-light-associated primary malignant melanomas of mucous membranes. J Clin Microbiol 46:3595–3598.

Goh S, Lindau C, Tiveljung-Lindell A, Allander T. 2009. Merkel cell polyomavirus in respiratory tract secretions. Emerg Infect Dis 15: 489–491.

Kassem A, Schopflin A, Diaz C, Weyers W, Stickeler E, Werner M, Zur Hausen A. 2008. Frequent detection of Merkel cell polyomavirus in human Merkel cell carcinomas and identification of a unique deletion in the VP1 gene. Cancer Res 68:5009–5013.

Katano H, Sato Y, Sata T. 2001. Expression of p53 and human herpesvirus 8 (HHV-8)-encoded latency-associated nuclear antigen (LANA) with inhibition of apoptosis in HHV-8-associated malignancies. Cancer 92:3076–3084.

Kuramochi H, Hayashi K, Uchida K, Miyakura S, Shimizu D, Vallbohmer D, Park S, Danenberg KD, Takasaki K, Danenberg PV. 2006. Vascular endothelial growth factor messenger RNA expression level is preserved in liver metastases compared with corresponding primary colorectal cancer. Clin Cancer Res 12:29–33.

Lemos B, Nghiem P. 2007. Merkel cell carcinoma: More deaths but still no pathway to blame. J Invest Dermatol 127:2100–2103.

Pectasides D, Pectasides M, Economopoulos T. 2006. Merkel cell cancer of the skin. Ann Oncol 17:1489–1495.

Ridd K, Yu S, Bastian BC. 2009. The presence of polyomavirus in non-melanoma skin cancer in organ transplant recipients is rare. J Invest Dermatol 129:250–252.

Sastre-Garau X, Peter M, Avril MF, Laude H, Couturier J, Rozenberg F, Almeida A, Boitier F, Carlotti A, Couturaud B, Dupin N. 2009. Merkel cell carcinoma of the skin: Pathological and molecular evidence for a causative role of MCV in oncogenesis. J Pathol 218: 48–56.

Sharp CP, Norja P, Anthony I, Bell JE, Simmonds P. 2009. Reactivation and mutation of newly discovered WU, KI, and Merkel cell carcinoma polyomaviruses in immunosuppressed individuals. J Infect Dis 199:398–404.

Shuda M, Feng H, Kwun HJ, Rosen ST, Gjoerup O, Moore PS, Chang Y. 2008. T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. Proc Natl Acad Sci USA 105:16272–16277.

Shuda M, Arora R, Kwun HJ, Feng H, Sarid R, Fernandez-Figueras MT, Tolstov Y, Gjoerup O, Mansukhani MM, Swerdlow SH, Chaudhary PM, Kirkwood JM, Nalesnik MA, Kant JA, Weiss LM, Moore PS, Chang Y. 2009. Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors. Int J Cancer 125:1243–1249.

Skelton HG, Smith KJ, Hitchcock CL, McCarthy WF, Lupton GP, Graham JH. 1997. Merkel cell carcinoma: Analysis of clinical, histologic, and immunohistologic features of 132 cases with relation to survival. J Am Acad Dermatol 37:734–739.

Van Gele M, Boyle GM, Cook AL, Vandesompele J, Boonefaes T, Rottiers P, Van Roy N, De Paepe A, Parsons PG, Leonard JH, Speleman F. 2004. Gene-expression profiling reveals distinct expression patterns for Classic versus Variant Merkel cell phenotypes and new classifier genes to distinguish Merkel cell from small-cell lung carcinoma. Oncogene 23:2732–2742.

zur Hausen H. 2008. A specific signature of Merkel cell polyomavirus persistence in human cancer cells. Proc Natl Acad Sci USA 105: 16063–16064.

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Short Communication

Virus Detection Using Viro-Adembeads, a Rapid Capture System for Viruses, and Plaque Assay in Intentionally Virus-Contaminated Beverages

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SUMMARY: Intentional contamination of beverages with microbes is one type of bioterrorist threat. While bacteria and fungus can be easily collected by a centrifuge, viruses are difficult to collect from virus-contaminated beverages. In this study, we demonstrated that Viro-Adembeads, a rapid-capture system for viruses using anionic polymer-coated magnetic beads, collected viruses from beverages contaminated intentionally with vaccinia virus and human herpesvirus 8. Real-time PCR showed that the recovery rates of the contaminated viruses in green tea and orange juice were lower than those in milk and water. Plaque assay showed that green tea and orange juice cut the efficiency of vaccinia virus infection in CV-1 cells. These results suggest that the efficiency of virus detection depends on the kind of beverage being tested. Viro-Adembeads would be a useful tool for detecting viruses rapidly in virus-contaminated beverages used in a bioterrorist attack.

Intentional contamination of beverages with microbes is a relatively easy way for terrorists to transmit microbes to anonymous persons and to induce a public panic. Although the few incidents of intentional viral contamination of drinks in Japan to date have not been real bioterrorist attacks, a similar act as a form of terrorism could be devastating. Therefore, an efficient and rapid detection system to detect microbes in contaminated drinks should be developed as an anti-bioterrorism tool. While bacteria and fungi can be easily collected by a centrifuge, viruses are difficult to collect from virus-contaminated beverages. An ultracentrifuge is a useful tool for virus collection in liquid samples, but not every laboratory is equipped with an ultracentrifuge. In addition, virus concentration with an ultracentrifuge usually takes more than 3 h.

Viro-Adembeads (Ademtech, Pessac, France) are recently developed magnetic beads intended for capturing viruses in liquid samples. They are specifically designed to function in virus-containing cell culture media (1,2). An anionic polymer coating on the magnetic beads binds to the surface of virus particles electrically; the complex of virus and beads can then be collected using a magnet. In the present study, we examined the capacity of Viro-Adembeads to collect viruses in virus-contaminated beverages. We also investigated whether the kind of beverage tested affected the efficiency of virus detection in this manner.

To represent intentional contamination of beverages with viruses, we mixed a solution containing two viruses with beverages. Human herpesvirus 8 (HHV-8) and vaccinia virus (LC16m8) were collected as reported previously (3,4). Milk, green tea, water, orange juice, and barley tea were purchased from a convenience store in Tokyo. To create virus-contaminated beverages, we added 0.1 mL of virus solution containing 1×10^7 copies of HHV-8 or vaccinia virus into 0.9 mL of beverages in 1.5-mL tubes. Each virus-contaminated beverage was serially diluted ×10 with the beverage at each stage, to ×10,000. If a terrorist was to contaminate beverages with viruses in a food store, the period of incubation would probably be from 5 min to several days; for our sample incubation time, we incubated the contaminated beverages for 1 h. To collect viruses from virus-contaminated beverage samples, we then used Viro-Adembeads according to the manufacturer's instructions. Briefly, 25 μL of washed Viro-Adembeads solution was added to each 1-mL aliquot of a virus-contaminated beverage sample. After 20 min of agitation at room temperature, the Viro-Adembeads were collected with a magnet. The beads were then washed with PBS twice. DNA was directly extracted from the beads with a DNA extraction kit (DNeasy; Qiagen, Hilden, Germany). The DNA was dissolved in 100 μL of water. Virus copy numbers were measured with a TaqMan Real-Time PCR (Applied Biosystems, Foster City, Calif., USA) as previously described (5). To detect HHV-8, we amplified ORF-26 using a previously reported probe and primer set (6). To detect vaccinia virus, we used a consensus probe and primer set targeting the F2R region of orthopoxvirus as follows: forward primer 5′-gatctagttttcagcacggtggta-3′, reverse primer 5′-cagatatatgattggatgtagaacaccat-3′, and probe 5′-FAM-agaggtggaggaatttatagatgatggaagacaagtt-TAMRA-3′. The recovery rate was calculated by the retrieved virus copy number with Viro-Adembeads, divided by the input virus copy number in 1 mL of each sample. The results of real-time PCR showed that the amount of collected virus was proportionally reduced as the sample was diluted (Figure 1A and 1B). Copies of HHV-8 and vaccinia virus were reduced almost one-tenth per dilution. However, copies of HHV-8 in ×100, ×1,000, and ×10,000 dilutions of green tea, and vaccinia virus in the ×10 dilution of green tea were extremely reduced. Although 34% of input HHV-8 was detected in the water sample, other beverages such as milk, orange juice, and green tea demonstrated lower recovery rates (Figure 1C). The recovery rate of HHV-8 with Viro-Adembeads was 39% in culture media (RPMI 1640 medium supplemented with 10% fatal bovine serum) containing the same amount of HHV-8 to the ×10 dilution, suggesting similar efficacy of virus collection with Viro-Adembeads between water and culture

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