しており，その根本治療を早期に行うことは病状の改善と他の感染症併発の防止のために必要である。しかしながら，ARTを開始することにより日和見感染症の治療薬に加えて3剤以上の抗HIV薬を投与することになり，治療の複雑化と薬剤相互作用，副作用の発生リスクが高まることにも注意が必要である。さらには，急速な免疫の回復が病原体(抗原)に対する強い免疫応答を引き起こすことになり，日和見感染症の病態の悪化を招く可能性がある。このようなARTによる日和見感染症の悪化や出現を免疫再構築症候群(immune reconstitution inflammatory syndrome：IRIS)と呼んでいる。

ARTをどのタイミングで行うかは未解決の問題であるが，クリプトコックス髄膜炎ではIRISによって意識障害や脳機能の後障害を残す可能性があり，一般にいわれる早期のARTがよいかどうかは疑問が多い。筆者は，少なくとも初期のAMPH-Bを用いた治療中はクリプトコックス髄膜炎の治療に専念し，FLCZ治療にスイッチした段階で患者の状態をみながら治療開始することを提唱する。実際，クリプトコックス髄膜炎によるIRISについて検討したBicanicらの検討[1]では，ARTを開始した時期はクリプトコックス髄膜炎治療開始から44～47日後と報告している。また，この研究結果をもとにDHHSガイドライン[2]でも，ART開始を少し遅らせたほうがよい日和見感染症の1つとしてクリプトコックス髄膜炎を挙げている。ただし，クリプトコックス治療薬の副作用などの難治例を除いて，ARTを数ヵ月以上も延期することは他の合併症のリスクもあり推奨できない。IRISを起こさないことを目標にするのではなく，重篤なIRISを防ぎながらなるべく早期にARTを導入することが重要であり，IRISの発症を予測し適切なコントロールをすることを目指すべきであろう。

IRIS発症時の対処

クリプトコックス髄膜炎でのIRISでは発熱や頭痛に加えて，容易に意識障害や脳機能障害を惹起する。したがって，微熱のみといった軽微な場合を除き，早期に対処すべきである。第一選択は副腎皮質ステロイドホルモンの投与であり，プレドニゾロン換算で0.5～1.0mg/kgからそれ以上のデキサメタゾンを経静脈的に投与する。症状の改善をみながら，2～6週間かけて漸減終了を目指す。また，クリプトコックス髄膜炎の治療は可能であればAMPH-Bに戻すか，FLCZは400mg/日かそれ以上の量とすることを考慮する。

ステロイドでも改善傾向がみられなければ，ARTの一時中止を考慮すべきである。

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種が最も基本となる予防法です．なお，ワクチンの効果とは，「未接種で罹患した人のうち，接種していれば罹患しないで済んだのは何%か？」という数字です．

ワクチンの集団免疫効果

ワクチンには，本人への発症予防効果（直接的効果）以外に，ある程度の接種率であれば他人に感染させない集団免疫効果（間接的効果）があり，この間接的効果はインフルエンザでも報告されています[4]．インフルエンザを患者にうつす可能性を下げるためにも，医療従事者は毎年インフルエンザワクチンを接種するべきです．

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Question 3　B 型肝炎ワクチン

B 型肝炎ワクチンの接種を受けましたが，抗体ができません．どうしたらよいですか？

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Answer

B 型肝炎とワクチン接種

B 型肝炎は血液を介して感染する感染症としては，日本で日常的に遭遇し，感染した場合の影響が深刻な感染症です．

病原微生物は小型 DNA ウイルスで，HBs 抗原陽性者の血液に最大 10^{10}/mL と高濃度に存在し，この血液が付着した針刺しの場合，30%近い高確率で感染が成立します．感染した場合，1～4ヵ月の潜伏期の後，急性肝炎を発症し，1%弱では激症化し死亡するリスクもあります．免疫不全状態や幼小時に感染すると慢性感染となります．最近はジェノタイプ A のウイルスが増加してきており[1]，このウイルスでは成人でも 10%程度で慢性化するといわれています．

このように日常的に存在し，感染リスクの高い血液媒介感染症であるため，医療に関わる従業者は B 型肝炎のワクチン接種が勧奨されています．ワクチンは3回の接種で1シリーズとなり，1回目の1ヵ月後，6ヵ月後に再接種を行い，3回目の

接種後1ヵ月以上経ってから抗体検査を行い，HBs抗体が10 IU/mL以上となれば抗体を獲得したとみなされます[2]．

抗体を獲得できない場合

接種者の90～95%では抗体を獲得しますが一部で抗体ができない場合があります．この場合もう1シリーズの接種を行うことで，再接種者の50%ほどで抗体を獲得するとされています．抗体獲得率の向上の方法として製造メーカーを変えてみること，1回接種量を倍量とすること，10分の1量を皮内接種し残りを皮下または筋肉注射すること[3]などが報告されています[2]．後二者の方法は日本で承認されているワクチンの用法・用量にない方法ですので，実施者の責任において行うことになります．なお，B型肝炎ワクチンは白色懸濁液で，白濁した部分が抗原となる成分ですので，接種にあたっては液を十分混合し，沈殿が生じる前に注射器内に接種量を充填し，注射器の薬液すべてを注射することが肝要です（接種液量ばかりに気を取られて充填に時間をかけると，バイアル内や注射器内に成分が残存し，上澄のみを接種することになってしまいます）．

2シリーズのワクチン接種でも抗体を獲得しなかった場合は，3シリーズ目を行ってもその効果は限定的ですので一般的には推奨されませんが，接種ができないわけではありません．

いったん抗体陽性となっても，時間が経つと抗体価が下がり，抗体価が測定感度以下となる場合があります．日本ではワクチンの再接種を行うこともありますが，米国[4]やヨーロッパ[5]では免疫不全がない場合は，いったん抗体陽性となっていれば抗体価が下がった状態でウイルスに曝露された場合，顕性の肝炎を発症することはないため，再接種は推奨されていません．

HBs抗体陰性者がHB陽性血に曝露した場合はHBs抗体高力価のガンマグロブリン製剤の接種と平行して，HBワクチンの接種を開始することになりますが，2シリーズ以上のワクチン接種を行っても抗体を獲得していない場合は，曝露後のワクチンの効果が限られています．CDCガイドライン[4]ではこのような場合，ガンマグロブリンの接種を曝露直後と1ヵ月後の2回行うことを推奨しています．ただし2回目の接種については日本では労働災害保険の給付対象とならないものと思われます．

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137 サイトメガロウイルス感染症
Cytomegalovirus Infection

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定義・概念：サイトメガロウイルス(cytomegalovirus：CMV)による感染症である。CMVは感染者の唾液などの体液を介してヒト-ヒト感染を起こし，その後生体内への潜伏感染となる。初感染によるウイルスの全身播種に伴う急性感染症と，潜伏感染が生体の免疫不全によって活性化して生じる日和見感染症，妊娠母体が急性感染を起こすことによる新生児の先天感染の病型がある。

病態生理：CMVはβヘルペスウイルスに属し，ヒトに感染するウイルスのなかで最大のサイズを有する。他のヘルペスウイルスと同様，いったん感染すると持続感染(潜伏感染)となり，白血球や内皮細胞を始め種々の生体細胞内で増殖可能なウイルスが存在し続ける。わが国では成人までにほとんどが感染するとされていた。わが国のHIV感染者では感染経路にかかわらず95%以上が既感染となっているが，妊娠可能年齢の女性の感染率は70～50%台まで低下してきていることが報告されている。

小児期では感染しても無症候から他の急性ウイルス感染症と区別できない形で経過するが，思春期以降では伝染性単核球症様の病態を生じる。妊娠中の女性が感染すると児に先天性サイトメガロウイルス感染症を発症するリスクがある。

既感染者が細胞性免疫不全状態となると，CMVの再活性化により，肺炎，脳・髄膜炎，網膜炎，副腎炎，消化管潰瘍などさまざまな病態を発症する。

臨床症状・経過・予後

1. 急性感染

急性感染は既感染者の唾液・尿・母乳などの体液の曝露によって発症すると考えられており，感染から20～60日程度の潜伏期の後，急性熱性症状を発症する。その程度は無症候から高熱を示すものまでさまざまであるが，とくに幼少児では無症候性に経過する場合が多いと考えられている。思春期に入ると伝染性単核症様の急性症状を呈する割合が高くなる。症状はEBウイルスによる伝染性単核症と類似しており，発熱，皮下リンパ節の腫脹，軽度から中程度までの肝機能障害，異型リンパ球の出現などである。後述の免疫不全がなければ2～6週間の経過で自然軽快する。しかし前述のとおり，他のヘルペスウイルス同様にCMVは潜伏感染状態となり，生体から排除されることはない。

新生児が経産道や母乳によって出生早期にCMV急性感染症を発症することがある。通常出産の児であれば無症状で経過することが多いが，早産児・低出生体重児の場合，急性感染症状やCMV肺炎・肝炎を発症するリスクがある。このためリスクが高い児ではCMV陽性の母親からの母乳を避けるなどの注意が必要である。

2. 免疫不全に伴う臓器感染症

HIV感染症や先天性免疫不全，抗腫瘍薬，免疫抑制薬の使用により細胞性免疫の低下が持続する病態になると，CMVの再活性化が起こり，種々の臓器病変を発症する。基礎となる免疫低下に応じて，発症しやすいCMV病変がある。造血系の悪性疾患ではCMV肺炎や消化管病変(食道から大腸まで)，HIV感染症ではCMV網膜炎，消化管病変，髄膜炎が起こりやすいことが知られている。いずれも難治性で早期治療が行われないと恒久的な臓器障害を残したり，致死的転帰となる割合が高い。

3. 先天感染(子宮内感染)

妊娠中の母体がCMVの急性感染を起こした場合，5%程度の児で何らかの症候がみられる。出血斑，肝脾腫と黄疸，小脳症，脳の石灰化，発育遅滞，運動障害，脈絡網膜炎などが知られており，重症の場合は意識障害，けいれん，呼吸障害から数週以内に死にいたる。

出生児無症候であっても，運動神経障害，聴覚・視覚障害が後になって明らかになる場合もあるため，生後数年間は経過観察と早期の対処が重要である。

診断基準：CMV感染症の診断は特徴的な臨床症状に加えて，CMVを検出することで行われる。PCRによるCMVの検出，半定量的なサイトメガロウイルス抗原血症(CMV-アンチゲネミア)の検出がいずれの病型でも確定診断に有用である。組織診や細胞診によって特徴的な巨細胞封入体の確認や，モノクローナル抗体による病変組織におけるウイルスの確認は最も確実な診断方法である。このほか急性感染と先天性感染ではIgM型のCMV抗体の検出，IgG型抗体の4倍以上の上昇なども診断の参考となる。

鑑別としては急性感染ではEBウイルスによる伝染性

表　CMV 治療薬と特徴

治療薬	ガンシクロビル	バルガンシクロビル	ホスカルネット
適応	後天性免疫不全症候群，臓器移植，悪性腫瘍における CMV 感染症		後天性免疫不全症候群患者の CMV 感染症 造血幹細胞移植患者における CMV 血症およびCMV感染症
小児への投与	小児に対する安全性は確立されていない 長期投与による発癌性および生殖毒性の可能性があることを慎重に考慮		小児に対する安全性は確立されていない 歯あるいは骨への沈着が成人より小児で多いことが予想される
治療投与量(1日)	5 mg/kg×2 回	900 mg×2 回(成人)	90 mg/kg×2 回
維持投与量(1日)	5 mg/kg×1 回	900 mg×1 回(成人)	90 mg/kg×1 回
投与方法	点滴静注	経口	点滴静注(十分な補液)
とくに注意すべき副作用	骨髄機能抑制 腎機能障害	同左	腎機能障害 電解質異常(Mg↓，Ca↓，K↓など) 骨髄機能抑制
備考	初期は隔日に血液検査	定期的な血液検査	初期は隔日に腎機能検査 点滴ごとに 0.5〜1 L の生理食塩液を補液

単核症，HIV やその他のウイルス感染の急性感染などがあげられる。免疫不全の場合はさまざまな日和見感染症の鑑別が必要になる。先天感染では，他の TOR(C)H などの先天感染症を鑑別する。

治療方針・治療基準：CMV の治療薬としては，Ganciclovir とそのプロドラッグである Valganciclovir，Foscarnet のみが日本で使用できる薬剤である。いずれも造血機能抑制や腎障害，電解質異常など強い副作用のある薬剤であり，免疫不全に伴う CMV 感染症・CMV 血症のみが適応となっている。治療薬の概要を表に示した。急性感染では髄膜炎など重篤な病態がみられない限り，抗ウイルス薬の適応とはならない。

免疫不全の場合は基本的に治療が必要であるが，HIV 感染症とそれ以外では対処がやや異なる。前者では病態をみながら治療を行うが，後者では血中の CMV ウイルスが検出された場合は明らかな臓器病変がみられなくても早期の治療開始が肝要とされている。いずれの薬剤も小児への投与に関する経験は少なく，治療を行う場合には十分な検討とインフォームド・コンセントが必要である。

Key Words：ヘルペスウイルス，免疫不全，先天感染，ガンシクロビル，ホスカルネット

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特集 おさえておきたい感染症と感染予防ケア

よく遭遇する感染症と感染対策，看護ケア

呼吸器感染症と感染対策 ②結核

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結核の概念と特徴

結核は空気感染で伝播する。患者が咳やくしゃみ，会話をする際に飛散した飛沫中の水分が蒸発し軽くなり，飛沫核となった結核菌を肺内に吸入することにより感染が成立する。

結核は感染と発病とは別に考えなくてはならず，肺内に吸入されても諸免疫機構がはたらき，実際には結核菌に曝露した人のうち半分程度に感染が成立し，そのうち10～20％の人が発病する。発病する人の約80％は感染後２年以内に，残りは数年から数十年後に発病する。

結核は感染症法の２類感染症に分類され，感染症法第12条より医師は結核と診断した患者があった場合にはただちに最寄りの保健所に届け出なければならない。患者の死亡後に結核と判明した場合，あるいは結核と診断された直後に死亡した場合にも届出を行う必要がある。また，同法第53条により病院管理者は結核患者が入院または退院したときは７日以内に保健所長に届出を行う。

結核の疫学

わが国における2011年の全結核患者数は22,681人で，人口10万対罹患率は17.7，そのうち菌喀痰塗抹陽性肺結核者数は8,654人，喀痰塗抹陽性罹患率は人口10万対6.8であった[1]。低下傾向ではあるものの，諸先進国と比べると罹患率は２～５倍とまだまだ中蔓延国といえる。

特徴の１つとしては，70歳以上の高齢者結核が半数以上を占めるようになっていることである。その約半数は呼吸器症状がなく，微熱，全身倦怠感，食欲不振，体重減少といった症状のみであり，発見の遅れにつながることがあるので注意が必要である。

また，わが国の医療従事者における結核罹患率は一般人口より高いことが知られており，とくに看護師，臨床検査技師の罹患率は他職種の同年齢層の罹患率と比較して高い[2]。

一方，病院・診療所・老人保健施設などの医療施設における結核集団感染発生は事業所における発生に続き，約18％を占めている（図１）[3]。結核集団感染の定義は同一の感染源が，２家族以上にまたがり，20人以上に結核を感染させた場合をいう。ただし，発病者１人は６人が感染し

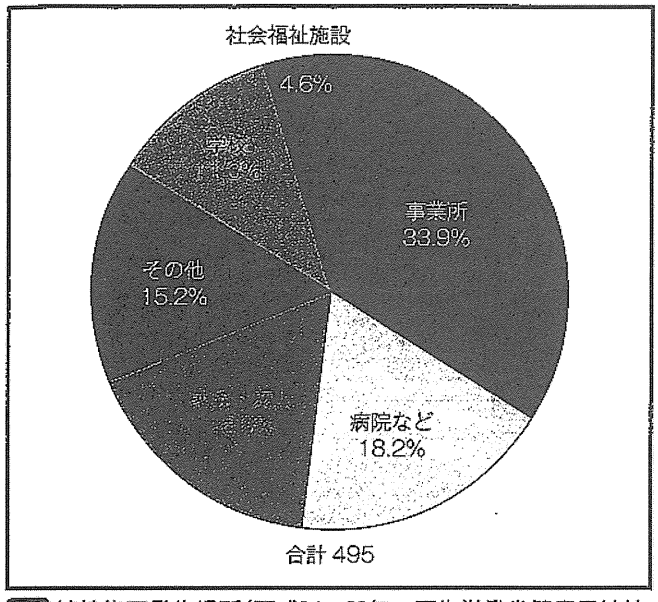

図1 結核集団発生場所(平成14～23年，厚生労働省健康局結核感染症課調査より)

たものとして感染者を計算する。

結核の感染対策

結核の院内感染防止は多方面からの方策をとらなければならない。

1 ▶▶▶早期発見

まずは感染源となる結核患者の診断を早期に発見することが院内伝播を予防する第一歩である。感染源になり得る結核とは肺結核および喉頭結核が代表である。とくに喀痰塗抹陽性例では感染性が高い。

また，結核性胸膜炎や粟粒結核でも肺実質に病変をみとめ，喀痰から結核菌が排出されていれば感染源となり得る。2週間以上続く咳では積極的に胸部X線写真を撮り，異常があれば必ず抗酸菌検査を行う。

高齢者や免疫抑制状態にある患者(悪性腫瘍，糖尿病，免疫抑制剤により治療中の患者など)，HIV感染者などの結核のハイリスクグループでは，咳のほかにも痰，血痰，胸痛，全身倦怠感，発熱などをみとめた場合には常に念頭に結核の存在をおき，これらの検査を行う[4]。

その他，外来患者で咳が激しいものは外科用マスクを着用させ(そのような症状があれば申し出るよう表示も必要)，他の待合室に隔離し，他患者との接触をできるだけ避ける。救急外来や呼吸器内科外来などでは陰圧室が1つはあることが望ましい。このような患者では優先的診療(トリアージ)を行う体制を整備しておくことも重要である。

また，外来で結核が強く疑われて採痰する場合，陰圧室がない場合は専用の採痰ブースにて採痰する。採痰ブースが設置できない場合は人のいない風通しのよい場所で行う。

2 ▶▶▶患者発生時の対応

結核と診断された(または疑う)患者は陰圧個室に隔離し，保健所に届出を行い，治療を開始する。また，喀痰塗抹で抗酸菌陽性であるが，胸部X線所見上は非結核性抗酸菌症が疑われる，肺外結核の診断はついているが，胸部の精査は行っていないなどといった場合も，培養，PCR，胸部CTなどで肺結核が除外されるまでは個室収容が安全である。

陰圧個室がない場合は，転院可能な患者は転院まで，基礎疾患が重篤で転院できない者は転院できるようになるまで，ひとまずは通常の個室へ隔離する。その際，空調設備が再循環式の場合(病室または特定区域からの排気の一部を循環させて，吸気の一部に用いる方式)でHEPAフィルターが備えられていない環境では，空調を止める必要がある。

また，そのような場合，簡易式のHEPAフィルター内蔵空気清浄機を使用する。できればこのような事態も想定して結核病床のない一般病院でも1～2室の全排気方式の隔離室を備えていることが望ましい。

患者が入室している陰圧個室は差圧計またはスモークテストなどで毎日陰圧を確認する。

空調設備の具体的な内容は「平成20年度厚生労働科学研

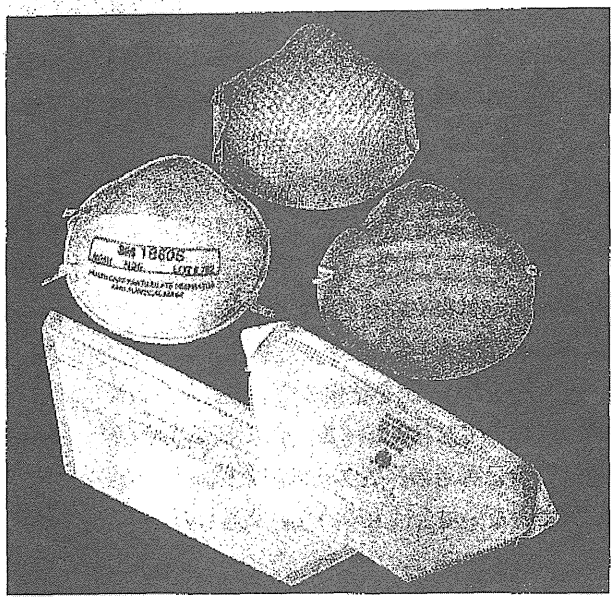

図2 種々のN95マスク

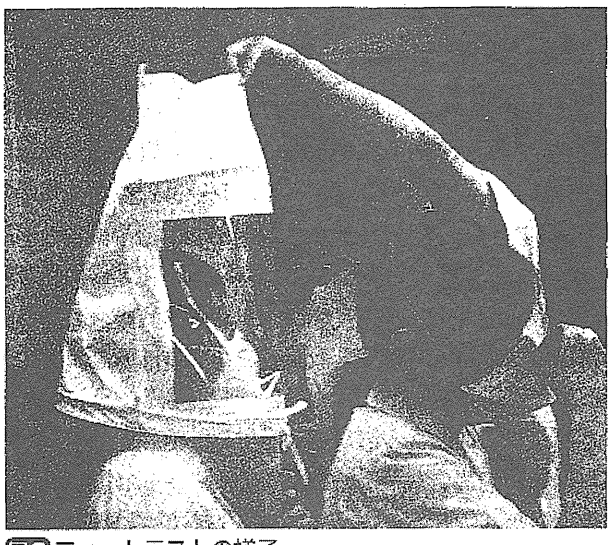

図3 フィットテストの様子

表1 1時間あたりの換気回数と空気中の汚染物質(飛沫核)の除去効率

換気回数	空気中の汚染物質(飛沫核)を99%または99.9%除去するのに要する時間(分)	
	99%	99.9%
2	138	207
4	69	104
6	46	69
12	23	35
15	18	28
20	7	14
50	3	6
400	<1	1

(Centers for Disease Control and Prevention: Guidelines for preventing the transmission of Mycobacterium tuberculosis in Health-care settings, 2005. MMWR 2005 ; 54(No. RR-17). より)

究費補助金(新興再興感染症研究事業)我が国における一類感染症の患者発生時の臨床的対応に関する研究，分担研究報告書，結核を想定した感染症指定医療機関の施設基準に関する研究平成21年3月」を参照いただきたい。

病室に入る前には，職員および面会者はN95マスクを着用する(図2)。自分に合ったサイズのマスクを着用し，きちんと着用できているかの確認をフィットテストにて行うことが重要である(図3)。

やむなく病室外で検査などを行わなければならない場合は，患者にはN95マスクではなく外科用マスクを着用してもらう。検査室やエレベータあるいは廊下では可能な限り他の患者と接しないルートあるいは時間帯を設定しておく。また，検査室や関係部署には空気感染対策が必要である旨を伝達しておかねばならない。同様に，検査などの医療関係者が病室に入る場合，空気感染対策中であることがわかる表示を院内共通の認識事項として周知しておくことも伝播を予防するためには重要である。

患者が使用した食器は通常の処理を行い，リネン類も標準予防策に準じて洗濯を行う。患者家族にも食器やリネンの取り扱いについては特別に処理する必要はないことを十分説明する。血圧計，聴診器などは専用にする必要はない。喀痰は専用のごみ箱を設置し，室内でビニール袋などを用いて密閉して感染性廃棄物として廃棄する。2類感染症である結核の治療および検査で使用した後のものは感染性廃棄物となるが，日常生活から出る一般のゴミは通常どおり処理する。

患者が退去した後は，部屋の入口扉を閉め，空気中の飛沫核が除去されるまで空室にする。除去されるまでの時間は1時間あたりの換気回数による(表1)。通常1時間あた

り12回の換気では，約30分で99.9％の飛沫核を除去できる[5]。

部屋の清掃やディスポーザブルでない医療機器の消毒，滅菌についてはガイドラインや院内マニュアルに従い通常どおり行う。

3 ▶▶▶患者発生後の対応

1）接触者健診

医療施設で発生した場合でも，自施設のみで事後対処せず，保健所長の指導のもと協働して行う。その際，濃厚接触者，非濃厚接触者，非接触者の判断をすることになるが，面会の家族を除いた考えられる医療施設内の濃厚接触者とは，同室患者，主治医，担当看護師，気管支鏡検査や採痰を担当した医師，看護師，検査技師があげられる。同室患者の主治医と担当看護師，X線技師などは非濃厚接触者に入るであろう。接触者健診の詳細は『感染症法に基づく結核の接触者健康診断の手引き改訂第4版』を参照いただきたい。

2）結核早期発見のための職員健康管理

日本結核病学会予防委員会では雇い入れ時の健康診断に際しては法令に定められた検査項目のほか，クオンティフェロン(QFT)検査の実施を推奨している。とくに結核患者と常時接する職場(結核病棟など)で強く奨めている。コスト面で難しい面もあるが，もしQFT検査を行わない場合，明らかに結核患者との接触歴がない者はベースライン陰性として扱う。

曝露後，本検査で陽性化したものには化学予防を行うが，専門医に相談のうえ，服用を開始するのが望ましい。そのほか，毎年の健康診断は必ず受診する体制が必要である[6]。

4 ▶▶▶その他

1）細菌検査

痰や培養菌などを取り扱う際は安全キャビネット内で操作し，できれば検査室は陰圧の環境とするのがよい。

2）気管支鏡検査

結核が疑わしいがどうしても喀痰で診断がつかない場合，やむなく気管支鏡を施行することもあり得るが，その際にはできるだけ最後に検査を組み入れ，陰圧の気管支鏡室が確保できなければ，簡易式のHEPAフィルター内蔵空気清浄機を設置するなどの工夫が必要である。

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Journal of Medical Virology 84:479–486 (2012)

Detection of Active Human Cytomegalovirus by the Promyelocytic Leukemia Body Assay in Cultures of PBMCs From Patients Undergoing Hematopoietic Stem Cell Transplantation

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A novel detection system was established previously for cells infected with the human cytomegalovirus (HCMV) in vitro that utilizes the unique IE1-dependent nuclear dispersion of promyelocytic leukemia (PML) bodies early in the HCMV replication cycle. This assay system, designated "the PML assay," makes use of the GFP-PML-expressing cell line SE/15, and allows real-time monitoring of infected cells by fluorescence microscopy without any staining procedures. A rapid and quantitative drug susceptibility testing was developed for low-titer clinical isolates propagated in fibroblasts in vitro. The present study sought to exploit the PML assay for evaluating in vivo status of HCMV without virus isolation. Progeny viruses were detected directly from peripheral blood mononuclear cells (PBMCs) infected in vivo obtained from hematopoietic stem cell transplantation recipients. The overall positivity of the PML assay tended to correlate with the levels of genomic DNA. Direct phenotypic susceptibility testing detected one ganciclovir (GCV)-resistant case among 19 samples, which was confirmed further by genomic and plaque reduction assays. However, in another patient with the sequence-proven mutant confirmed by sequencing, the progeny viruses exhibiting GCV-resistance were not detected. Studies on the isolated virus from the latter patient suggested the possibility that replication efficiency may differ between PBMCs and lesions infected in vivo, which may hamper the detection of GCV-resistant viruses by the PML assay, at least in this case. Taken together, the PML assay is sufficiently sensitive to monitor replication-competent HCMV directly from PBMCs infected in vivo, and provides a novel tool for comparing the characteristics of HCMV strains infected in vivo. ***J. Med. Virol. 84:479–486, 2012.***

KEY WORDS: PML assay; GFP-PML; HCMV; clinical isolate; drug susceptibility testing; hematopoietic stem cell transplantation

INTRODUCTION

Despite recent advances in antiviral therapy, human cytomegalovirus (HCMV) remains a common viral pathogen that causes problematic infections, especially in patients undergoing hematopoietic stem cell transplantation and solid organ transplantation. The diagnosis and monitoring of HCMV in hematopoietic stem cell transplantation and solid organ transplantation recipients have advanced rapidly in the last two decades [Razonable et al., 2002; Baldanti et al., 2008], which has allowed the development of

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prophylactic or preemptive therapies for HCMV infection and disease [Griffiths and Boeckh, 2007]. The widely used HCMV diagnostic assays include the detection of pp65-positive leucocytes in antigenemia assays and quantitative PCR assays of viral DNA in blood [The et al., 1990; Grossi et al., 1995; van der Meer et al., 1996], both of which are based on the presence of virus or viral products in blood irrespective of the infectivity. Although pp65-guided preemptive therapy is highly effective in the management of HCMV infection and disease [Boeckh et al., 1996; Kanda et al., 2001; Asano-Mori et al., 2005], and is believed to reflect the activity of HCMV in vivo, insufficient sensitivity has been reported occasionally in patients with HCMV-associated gastrointestinal disease or HCMV retinitis [Boeckh and Ljungman, 2009]. On the other hand, genomic DNA evaluation by quantitative PCR is a highly sensitive method, although increased viral loads may include both replication-competent and -incompetent viruses and/or debris from infected cells [Razonable et al., 2002; Baldanti et al., 2008]. To improve patient management, rapid and accurate determination of drug susceptibility is important to distinguish apparently reduced sensitivity against antivirus drugs caused by severe immunosuppression. Some paradoxical phenomena, such as dissociation between antigenemia and the viral load, have been reported [Gerna et al., 2003]. Analysis of HCMV infectivity in vivo is important for understanding the molecular basis of these inconsistent phenomena, concomitant with currently available clinical testing. Although the shell vial assay is an alternative method for evaluating replication-competent viruses [Gleaves et al., 1984; Griffiths et al., 1984; Patel et al., 1995], it is labor-intensive for monitoring active HCMV in clinical specimens.

A novel detection system was established previously for cells infected with HCMV in vitro that makes use of SE/15 cells stably expressing GFP-PML [Ueno et al., 2006; Ueno and Ogawa-Goto, 2009]. In the nucleus of these cells, HCMV deposits its genome adjacent to promyelocytic leukemia (PML) bodies and starts IE transcription, which leads to disruption of the PML bodies mainly through de-SUMOylation of PML protein by the function of IE1 protein [Korioth et al., 1996; Ahn and Hayward, 1997; Muller et al., 1998]. Hence, visual assessment by fluorescence microscopy is sufficient for detection without the necessity for any further procedures, such as fixation and staining. The assay can detect progeny viruses of not only laboratory strains but also clinical isolates. These features permit continuous tracing over the time course of a single culture, and are particularly advantageous for clinical specimens, the amounts of which are often limited. Thus, it is expected that the use of GFP-PML-expressing cells will provide a quantitative method for monitoring replication-competent viruses as an alternative to the shell vial assay.

In the present study, a protocol for direct detection of HCMV progeny viruses is presented for evaluation of peripheral blood mononuclear cells (PBMCs) infected in vivo from patients undergoing hematopoietic stem cell transplantation, which enables susceptibility tests for anti-HCMV drugs without virus isolation procedures. In this article, the detection system using GFP-PML-expressing cells is designated the PML body assay ("the PML assay"), and positive cells exhibiting diffuse GFP-PML in their nucleus are designated "PML-positive" cells, which were triggered by infection of progeny viruses during in vitro cocultures.

MATERIALS AND METHODS

Patients and Clinical Specimens

The present prospective study was approved by the ethical committees at Saitama Medical Center of Jichi Medical University and Toranomon Hospital. After informed consent was obtained, 25 whole blood specimens from 20 individuals who underwent allogeneic hematopoietic stem cell transplantation from an HLA-matched or -mismatched donor at Saitama Medical Center of Jichi Medical University or Toranomon Hospital were evaluated. The patients were diagnosed as positive in HCMV antigenemia tests by the C7-HRP or C10/C11 method during routine surveillance. The patient characteristics are summarized in Table I.

Blood specimens (~15 ml) were aliquoted into three parts, comprising 200 μl for virus isolation in fibroblasts, 200 μl for DNA extraction and the remaining volume for PBMC preparation. PBMCs were prepared using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions, and cultured in fibronectin-coated 24-well plates at a density of 0.6–2 × 10^6 cells/well. DNA was extracted from 200 μl of whole blood using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany). Quantitative PCR of the genomic HCMV DNA was performed as described previously [Leruez-Ville et al., 2003].

TABLE I. Summary of Patients' Characteristics

Age, median (range)	50 (17–65)
Sex (male/female)	10/10
CMV serostatus before transplantation	
Recipient CMV-positive	19
Donor CMV-positive	10
Underlying disease	
AML	7
SAA	5
ATL	2
DLBCL	2
ALL	3
CML	1
Graft source	
Bone marrow	10
Peripheral blood stem cell	7
Cord blood	3
Donor type	
Matched related	3
Mismatched related	4
Unrelated	13

Cell Culture, Virus, and Reagents

The SE/15 cell line [Ueno et al., 2006] was used throughout this study. HCMV clinical isolates were propagated and titrated in human embryonic lung fibroblasts. Virus inoculums were prepared by ultrasonication of infected fibroblasts and stored in medium containing glycerol. Plaque reduction assays in fibroblasts or drug susceptibility tests estimated by SE/15 cells were carried out as described previously [Ueno et al., 2006]. Ganciclovir (GCV), foscarnet (FOS) (Sigma–Aldrich, St. Louis, MO) and cidofovir (a generous gift from Prof. E. De Clercq) were used as anti-HCMV drugs.

Protocol for In Vitro Infection of PBMCs by Clinical Isolates and Detection of Progeny Viruses by SE/15 Cells

PBMCs were prepared from EDTA-treated blood obtained from HCMV genome-negative volunteers and cultured in RPMI1641 medium containing 10% FBS. The protocol for the PML assay was determined using PBMCs infected in vitro as follows. PBMCs (4×10^5) were cultured in 24-well plates, and non-adherent cells were removed by washing with PBS. Subsequently, 200 μl of a clinical isolate inoculum was added and incubated for 2 hr. The cells were then extensively washed with PBS and cultured in RPMI1641 medium containing 10% FBS. At 3 days post-inoculation, SE/15 cells (5×10^4) were added after extensive washing with PBS, and maintained until 9 or 10 days post-inoculation. The numbers of PML-positive cells were counted daily by fluorescence microscopy. To characterize the cell types of the HCMV-infected cells, PBMCs infected in vitro were subjected to immunofluorescence analyses using antibodies against UL44 (Dako, Glostrup, Denmark) and CD14 (Novocastra Laboratories, Newcastle Upon Tyne, UK). UL44, an HCMV early gene antigen, was detected in the nucleus of CD14-positive cells (data not shown), confirming that monocytes/macrophages were the targets for the viruses. Among several reagents reported to be enhancers of HCMV replication in fibroblasts [West and Baker, 1990], dexamethasone (Dex) was found to be effective for progeny production in these cultures. Therefore, 5 μM Dex was used in the following experiments for cells infected in vivo.

Coculture of PBMCs Infected In Vivo With SE/15 Cells and Monitoring of Progeny Viruses

PBMCs ($0.6–2 \times 10^6$ cells/well) from allogeneic hematopoietic stem cell transplantation recipients were cultured in RPMI1641 medium containing 10% FBS. In Exp.0D, the coculture was started on the day of PBMC preparation and continued until day 6 or 7, while in Exp.3D, SE/15 cells were added on day 3 of PBMC culture and the culture was continued until day 9. During the cocultures, the numbers of PML-positive cells were monitored daily by fluorescence microscopy. In some experiments, cocultures were performed in the presence or absence of GCV or FOS.

Statistical Analysis

Fisher's exact test was used to compare the positive rates of the PML assay or virus isolation.

RESULTS

Detection of Progeny Viruses Produced From PBMCs Infected In Vivo

To test whether the PML assay detects progeny viruses produced by cells infected in vivo, PBMCs from patients with a variety of antigenemia levels after hematopoietic stem cell transplantation were used. Two series of experiments were carried out, designated Exp.0D and Exp.3D (Fig. 1A). In Exp.0D, the coculture started on the day of PBMC preparation, while in Exp.3D, SE/15 cells were added on day 3 of PBMC culture, and the numbers of PML-positive cells were monitored (Fig. 1A and B). In most cases, PML-positive cells began to appear from days 3 to 4 and peaked around day 5 in Exp.0D, while they peaked from days 7 to 9 in Exp.3D. Representative curves for Exp.0D and Exp.3D in the presence and absence of 5 μM Dex are shown in Figure 1C and D, respectively. More than 60% of the specimens tested responded to Dex treatment to various degrees.

The results of the PML assay together with the virus isolation are summarized in Table II. Overall, 20 of 25 (80%) samples were positive in either Exp.0D or Exp.3D. On the other hand, HCMV was successfully isolated in 11 of 25 (44%) samples using fibroblasts, and PML assays were positive in 10 of the 11 culture-proven HCMV-infected specimens. For comparison with the genomic DNA levels, the samples were categorized into two groups based on the copy numbers of the HCMV genome, namely low-grade ($<5 \times 10^3$ copies/ml) and high-grade ($\geq 5 \times 10^3$ copies/ml) groups. No viruses were isolated in the low-grade group, while viruses were successfully isolated from 64% of the high-grade group specimens.

In the high-grade group, 12 of 16 (75%) samples exhibited PML-positive cells in Exp.0D, which was significantly higher than the corresponding 2 of 8 (25%) samples for the low-grade group. Higher numbers of PML-positive cells were observed in the high-grade group in both Exp.0D and Exp.3D. However, a considerable number of high-grade group specimens exhibited undetectable or very low levels of PML-positive cells despite high levels of antigenemia and/or genome copy numbers. No significant difference was observed for the peak appearance day between the two groups.

Drug Susceptibility Test Using PBMCs Infected In Vivo

To establish a susceptibility test for anti-HCMV drugs in Exp.3D, PBMCs were cultured singly in the

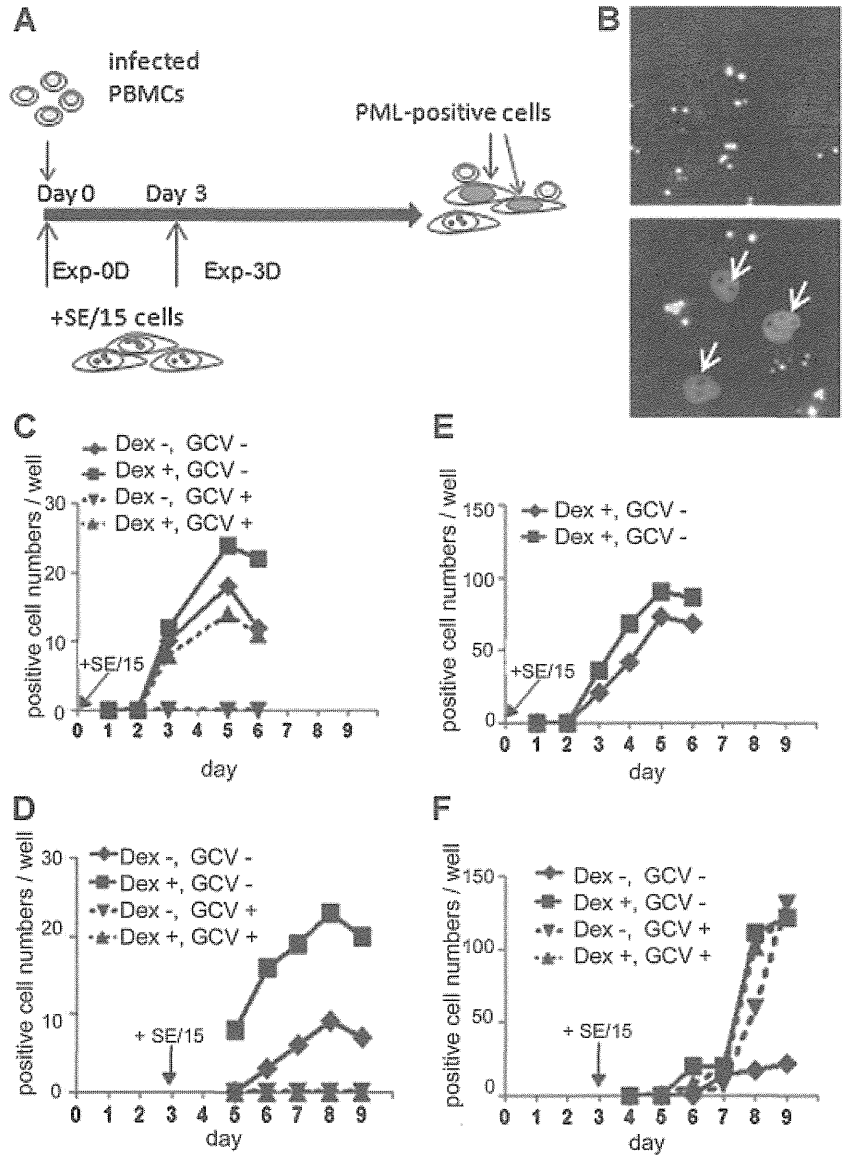

Fig. 1. Time-course profiles of progeny virus production from PBMCs obtained from hematopoietic stem cell transplantation recipients. **A**: Schematic representation of the PML assay using PBMCs infected in vivo obtained from patients after hematopoietic stem cell transplantation. **B**: Representative images of PML-positive cells (lower, shown by arrows) and PML-negative cells (upper) during the PML assay upon cocultures with the PBMCs infected in vivo. Images under fluorescence microscopy are shown. **C–F**: Exp.0D (C,E) and Exp.3D (D,F). In Exp.0D the coculture was started on the day of PBMC preparation. In panel C, the coculture was carried out in the presence or absence of GCV. In Exp.3D, SE/15 cells were added on day 3 of PBMC culture and the culture was continued until day 9 in the presence or absence of GCV. Representative curves for Exp.0D and Exp.3D in the presence and absence of 5 μM Dex are shown. C, D: #hsct-24; E, F: #hsct-14.

presence or absence of GCV for 3 days, followed by the start of coculture at day 3 (Fig. 1A). Addition of GCV inhibited the production of PBMC-derived progeny viruses in Exp.3D, but not in Exp.0D (Fig. 1C and D). The PML-positive cells in Exp.0D, even in the presence of GCV, were attributed to active viruses at blood sampling that had already passed through the phase of viral DNA replication, thereby escaping the inhibitory action of GCV that specifically targets viral DNA polymerase. Among the 19 samples tested for Exp.3D, six samples were determined to have apparent low sensitivity for GCV under the conditions used (Figs. 1F and 2A, Table III).

Genomic sequencing analyses revealed mutations conferring GCV-resistance in one patient (#hsct-17), who had been treated with GCV for more than 75 days. The mutations included C603W in the UL97 gene and T503I in the UL54 gene. Using an isolated virus strain from #hsct-17, an increased IC_{50} for GCV (21 μM) was confirmed, which was defined as a

TABLE II. Summary of the PML Assay With In Vivo Infected PBMCs and Virus Isolation

	The PML assay using in vivo infected PBMCs						Virus isolation in fibroblasts
	Exp.0D			Exp.3D			
	Positive % (positive/total)	Average peak appearance day	Median number of positive cells/ml blood (range)	Positive % (positive/total)	Average peak appearance day	Median number of positive cells/ml blood (range)	Positive % (positive/total)
Total	58 (14/24)	5.2	5.4 (0–68)	76 (19/25)	8.6	10.9 (0–141)	44 (11/25)
HCMV genome DNA							
Low grade ($5 \times 10^3 >$)[a]	25 (2/8)	5.5	0 (0–11)	63 (5/8)	8.3	3.0 (0–25)	0 (0/8)
High grade ($5 \times 10^3 \leq$)[a]	75** (12/16)	5.1	6.4 (0–68)	83 (14/17)	8.7	14.4 (0–141)	64*** (11/17)

[a]Copies/ml blood.
**$P < 0.05$ against low-grade group.
***$P < 0.005$ against low-grade group.

GCV-resistant level (Table III). On the other hand, neither apparent genomic mutations nor increased IC_{50} values were found in the other five cases, despite the production of progeny viruses in the presence of GCV (Table III). Several background factors reported to have impacts on the HCMV infection and disease had no bias for the apparent low susceptibility without genome mutations, including the donor HCMV serostatus before transplantation, acute graft-versus-host disease (GVHD) and relapse of antigenemia (data not shown).

A genomic mutation conferring GCV-resistance (L595S in the UL97 gene) was identified in another patient (#hasct-22), who had been treated with GCV for about 70 days. Although the antigenemia value jumped to over 7,000, the susceptibility test in Exp.3D was unable to detect progeny viruses produced from PBMCs exhibiting GCV-resistance (Fig. 2B). It was notable that a delayed peak (day 9 or later) with very low numbers of PML-positive cells was seen, which seemed to be uncorrelated with the extremely high levels of genomic DNA and antigenemia (Table III). In contrast, the IC_{50} value of the isolated virus in fibroblasts showed clear GCV-resistance (23.2 μM for GCV).

To further explore the reason for the discrepancy, the efficiencies of virus replication in vitro in different host cell species were addressed. When fibroblasts or PBMCs were infected with the same titers of the #hsct-22 isolate at the third passage, the replication speeds of #hsct-22 in PBMCs were apparently slower than those in a PBMC tropic control strain (isolate A) (Fig. 2C). Thus, an apparent inconsistency was observed for the PBMCs infected in vivo between the antigenemia levels/viral loads and progeny virus production estimated by the PML assay.

DISCUSSION

The hallmarks of the PML assay including high specificity, a simple procedure that enables sequential tracing, sensitive, and quantitative evaluation for low-titer clinical isolates have been verified by the previous study [Ueno et al., 2006]. The present study has demonstrated that the PML assay is sufficiently sensitive for direct monitoring of HCMV produced from PBMCs infected in vivo from hematopoietic stem cell transplantation recipients. In particular, the sequential tracing of the same culture has a clear advantage for analyzing the limited amounts of clinical specimens, where treatment by Dex enhanced viral replication in most of the PBMC specimens as in fibroblasts [West and Baker, 1990]. In addition, the diffuse nuclear pattern of GFP-PML has never been caused by infection with other herpes viruses including human HSV, VZV and HHV-6 (Ueno et al., unpublished observation), further confirming that the nuclear diffuse PML phenotype is HCMV IE-1 dependent [Ueno et al., 2006; Ueno and Ogawa-Goto, 2009].

In the present study, the overall positivity of the PML assay appeared to be correlated roughly with

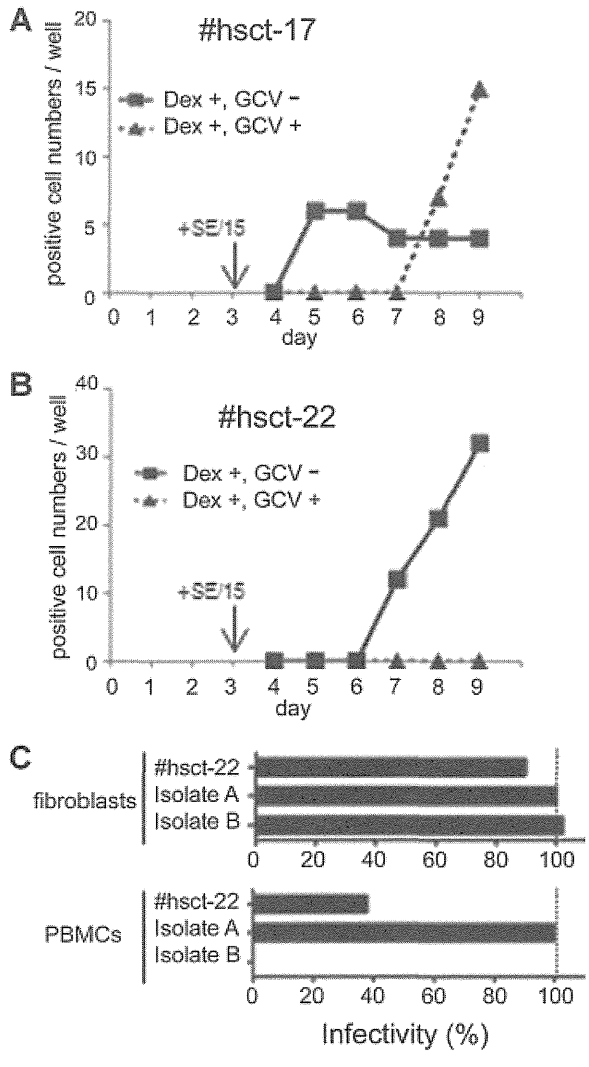

Fig. 2. Time-course profiles of progeny virus production and replication efficiency of sequence-proven GCV-resistant HCMV isolates. **A,B**: Progeny virus production was monitored in PBMCs from hematopoietic stem cell transplantation recipients who were diagnosed as being infected with GCV-resistant strains in a subsequent sequence analysis. SE/15 cells were added on day 3 of PBMC culture and the culture was continued until day 9 in the presence or absence of GCV. The data for Exp.3D are shown. A: #hsct-17; B: #hsct-22. **C**: The efficiencies of replication in fibroblasts and PBMCs were compared using the third passage isolate strain of #hsct-22, which had been propagated in fibroblasts. Isolate A: a positive control strain virus that can efficiently replicate in PBMCs; isolate B: a negative control strain virus that cannot replicate in PBMCs.

the levels of genomic DNA. Although the median numbers were increased modestly in the high-grade DNA group, the numbers of PML-positive cells were not well correlated with the DNA levels occasionally. Notably, there were several exceptional cases that showed apparent inconsistencies between the data from the PML assay and the viral loads. This tendency for inconsistency is elicited possibly by the different basis of the assays for evaluating the productive infectivity or the virus and/or viral products in blood [Razonable et al., 2002; Baldanti et al., 2008]. The levels of antigenemia or viral loads reflect the in vivo

TABLE III. Data of the PML Assay, Antigenemia, and Genomic DNA Levels, and Virus Characteristics Which Showed Low Susceptibility to GCV in the PML Assay

		HCMV Genome	The PML assay				Antigenemia			GCV treatment		Virus isolation	Genome analysis (blood)			IC50 (μM)	
					Susceptibility against												
Patient ID	Days post-transplantation at sampling	Copies/ml blood	Exp.0D Positive cells/ml blood	Exp.3D Positive cells/ml blood	GCV	FOS	Value[a] (day)	Assay method[b]	At sampling day	Duration (day)	In blood	UL54	UL97	GCV	FOS	CDV
#hsct-07	44	1.1×10^3	—	11.4	Low	S	261 (day 40)	A	+	16	−	—[c]	—[c]			
#hsct-09	63	5.8×10^3	—	17.0	Low		28 (day 61)	A	−	0	+	n.d.	n.d.	0.39	19	0.93
#hsct-12	163	1.7×10^4	33.3	10.9	Low	S	96 (day 159)	B	+	9	−	—	—			
#hsct-14	34	2.2×10^4	68.2	92.4	Low[#01]		79 (day 32)	A	+	14	+	n.d.	n.d.	0.33	64	1.3
#hsct-20	49	4.5×10^4	55.6	141.0	Low		14 (day 47)	B	+	14	+	n.d.	n.d.	0.46	178	1.8
#hsct-17	238	3.0×10^4	—	14.3	Low[#02]		61 (day 234)	A	+	77	+	T503I[d]	C603W[d]	21	81	4.4
#hsct-22	107	9.9×10^4	—	7.4	S[#03]		1,008 (day 107)	A	+	70	+	–	L595S[d]	23	92	2.8

S: sensitive; Low: low susceptibility assigned by the PML assay; n.d.: not determined.
#01, #02 and #03; time course profiles were shown in Figures 1F and 2A,B, respectively.
[a]Positive cell numbers/2 slides for data in C10/C11 assay or positive cell numbers/5×10^5 cells for data in C7-HRP assay were shown.
[b]A:C10/C11, B:C7-HRP.
[c]Sequence data showed no clear mutations conferring resistance, but partially contained unreadable ones probably due to heterogeneity of the template.
[d]Reported mutations conferring GCV resistance.

infectivity in the lesions, while the data in the PML assay are supposed to represent the productivity of replication-competent viruses derived from monocytes/macrophages infected in vivo. If this is the case, the dissociation between the antigenemia/viral loads and the PML assay may suggest that HCMV infected in vivo does not necessarily replicate with similar efficiency between PBMCs and the lesions.

The dissociation was particularly prominent in the sequence-proven GCV-resistant virus #hsct-22, which showed very low numbers of progeny viruses despite extremely high antigenemia or viral loads. Interestingly, slow replication in PBMCs was reproduced by in vitro experiments using simultaneously obtained blood culture isolates. Although virus tropism to leucocytes and/or endothelial cells can be affected by mutation of the genes encoding UL128–UL131A during in vitro culture [Akter et al., 2003; Hahn et al., 2004; Sinzger et al., 2008], a genomic sequence analysis revealed that the isolated virus at the fifth passage had no apparent mutations that led to amino acid substitutions or deletions in the UL128–UL131A genes compared with those of the blood sample (data not shown). To overcome the issues for these non-responsive samples, a detailed characterization of the virus is now being undertaken, including analyses of the genomic sequences of other genes and the clinical background information of the patients.

In most of the specimens tested in this study, direct phenotypic susceptibility testing under the conditions used appeared to be feasible, although further improvements are required. It is notable that some of the "apparent low-sensitivity" samples showed relatively high numbers of PML-positive cells, in contrast to the low numbers of PML-positive cells for the sequence-proven GCV mutants. Since the pathogenesis of HCMV infection is related to a number of interactions between HCMV and the host immune response, the host factors that can affect the numbers of PML-positive cells remain to be elucidated. However, a preliminary study revealed that several reported risk factors that impacts on HCMV infection had no bias for low GCV susceptibility, including the donor HCMV serostatus before transplantation, acute GVHD and relapse of antigenemia (data not shown). Further studies on the molecular basis of the cell type-specific preferential propagation of clinical strains may provide insights for better understanding of HCMV infection and disease.

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Novel monoclonal antibodies for identification of multicentric Castleman's disease; Kaposi's sarcoma-associated herpesvirus-encoded vMIP-I and vMIP-II

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ABSTRACT

Recent studies have indicated that vMIP-I and vMIP-II play important roles in the pathogenesis of Kaposi's sarcoma-associated herpesvirus (KSHV)-related diseases due to the effects of these proteins on vascularization. We developed monoclonal antibodies against KSHV-encoded viral macrophage inflammatory protein-I (vMIP-I) and vMIP-II to study these expression profiles and reveal the pathogenesis of KSHV-related diseases. The MAbs against vMIP-I and vMIP-II reacted to KSHV-infected cell lines after lytic induction. Both vMIP-I and the vMIP-II gene products were detected 24 h post-induction with 12-*O*-tetradecanoylphorbol-13-acetate until 60 h in the cytoplasm of primary effusion lymphoma cell lines. In clinical specimens, both vMIP-I and vMIP-II gene products were detected in the tissues of patients with multicentric Castleman's disease. On the other hand, only vMIP-II was detected in a subset of Kaposi's sarcoma. We concluded that these antibodies might be powerful tools to elucidate the pathogenesis of KSHV-related diseases.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a gammaherpesvirus originally identified in HIV-positive Kaposi's sarcoma (KS) tissues (Chang et al., 1994). KSHV is responsible for AIDS associated cancers such as Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (Cesarman et al., 1995; Schalling et al., 1995; Soulier et al., 1995). As is the case for all herpesviruses, KSHV has two life cycles, one latent and the other lytic. Lytic gene expression can be induced by the treatment of latently infected cells with chemical agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), sodium butyrate (Arvanitakis et al., 1996; Miller et al., 1997). It has been demonstrated that two KSHV-encoded chemokines, K6 (which encodes a vMIP-I) and K4 (which encodes a vMIP-II), are expressed in the course of lytic infection (Moore et al., 1996; Sun et al., 1999). Previous reports showed that both vMIP-I and vMIP-II induced Ca^{2+} signal transduction via certain chemokine receptors and the receptor-dependent migration of cells (Benelli et al., 2000; Chen et al., 1998; Endres et al., 1999; Kledal et al., 1997). In addition, in a chick chorioallantoic membrane assay, the both proteins showed strong angiogenic properties (Boshoff et al., 1997). However, little is known about the contribution of vMIPs to KSHV malignancy under physiologic conditions.

In this report, we generated new monoclonal antibodies against vMIP-I and vMIP-II, and confirmed the detection of both vMIP-I and vMIP-II in histological sections of tissues from MCD patients as well as in KSHV-infected PEL cell lines. In cases of KS, vMIP-II was detected, but not vMIP-I. These results suggest that the expression properties of vMIP-I and vMIP-II might be related to KSHV-associated diseases, and may even be involved in the generation of diseases. Thus, antiviral chemokine MAbs could potentially become useful tools for the diagnosis of KSHV-related diseases.

Materials and methods

Cells

Kaposi's sarcoma-associated herpesvirus-positive cell lines (BC-1, BC-3, BCBL-1 and TY-1 cells) and a negative cell line (BJAB cells) were

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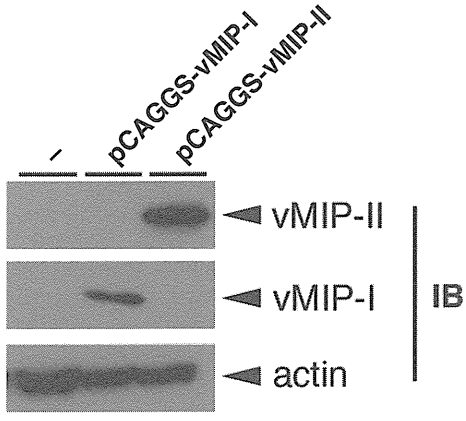

Fig. 1. Cross reactivity between anti-vMIP-I and anti-vMIP-II MAb. 293 T cells were transfected with either 2 μg of pCAGGS- vMIP-I or 2 μg of pCAGGS-vMIP-II plasmids. Forty-eight hours after transfection, the cells were harvested and expression of vMIP-I or vMIP-II was tested by Western blot analysis using the anti-vMIP-I or -vMIP-II MAb, respectively. Actin was also probed with anti-actin monoclonal Ab as a loading control.

obtained from the American Type Culture Collection (ATCC) (Manassas, VA). These cells were grown in RPMI 1640 (Nakalai Tesque, Inc., Kyoto, Japan) supplemented with 10 IU/ml penicillin G, 10 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) in a 5% CO_2 atmosphere. In addition, 293 T and 293/EBNA (Clontech) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Nakalai Tesque, Inc.) supplemented with 10 IU/ml penicillin G, and 10 μg/ml streptomycin, 10% FBS, and 200 mM L-glutamine.

Plasmids

In order to express vMIP-I and vMIP-II, the ORFs were cloned into the pCAGGS eukaryotic expression vector, and pCAGGS-vMIP-I and pCAGGS-vMIP-II were established. The plasmid vector, pCAGGS was kindly provided by Dr. J. Miyazaki of Osaka University (Niwa et al., 1991). Briefly, fragments including vMIP-I and vMIP-II ORFs were amplified by PCR using the following primer sets: vMIP-I-Met (5′-CGGTACCGAATTCTCCAGATGGCC-3′) and vMIP-I-Ter (5′-ACTCGAGAATTCTACTTGTCATCGTCGTCCTTGTAGTCGGAAGCTATGGCAGGCAG-3′); and vMIP-II-Met (5′-AGGTACCGAATTCAGTTATGGACACCAAGGGC-3′) and vMIP-II-Ter (5′-ACTCGAGAATTCTACTTGTCATCGTCGTCCTTGTAGTCGGAGCGAGCAGTGACTGG-3′). The PCR products were cloned into pCR2.1 (Invitrogen) and sequenced. After digestion with *Eco*RI, the fragments were ligated into the *Eco*RI site of the pCAGGS vector. Then, the DNA fragments encoding vMIP-I and vMIP-II were liberated by *Eco*RI, and were inserted into pCAGGS to generate the expression vectors pCAGGS-vMIP-I and -vMIP-II, respectively. vMIP-I (pGEX-vMIP-I) and vMIP-II (pGEX-vMIP-II) were also generated using PCR-based technology using BCBL-1 genomic DNA as a templete. The coding region, without a signal peptide, was amplified with vMIP-I-Eco (CAGAATTCGCGGGGTCACTCGTGTCG-3′), vMIP-I-Sal (CTGTCGACCGTCTAAGCTATGGCAGG-3′), vMIP-II-Eco (5′-CGGAATTCGCGTCCTGGCATAGACCG-3′), and vMIP-II-Sal (5′-GGGTCGACATTCTTCAGCGAGCAGTG-3′). The amplified vMIP-I and the vMIP-II fragments were digested with *Eco*RI and *Sal*I and inserted downstream of the GST coding of pGEX-5X-1 (GE Healthcare, Uppsala, Sweden) at the *Eco*RI and *Sal*I sites to construct pGEX-vMIP-I and pGEX-vMIP-II. To express a full-length and the deletion mutants of the GST-vMIP-I and GST-vMIP-II fusion protein, the genes for GvM1-Full, GvM1-D1, GvM1-D2,

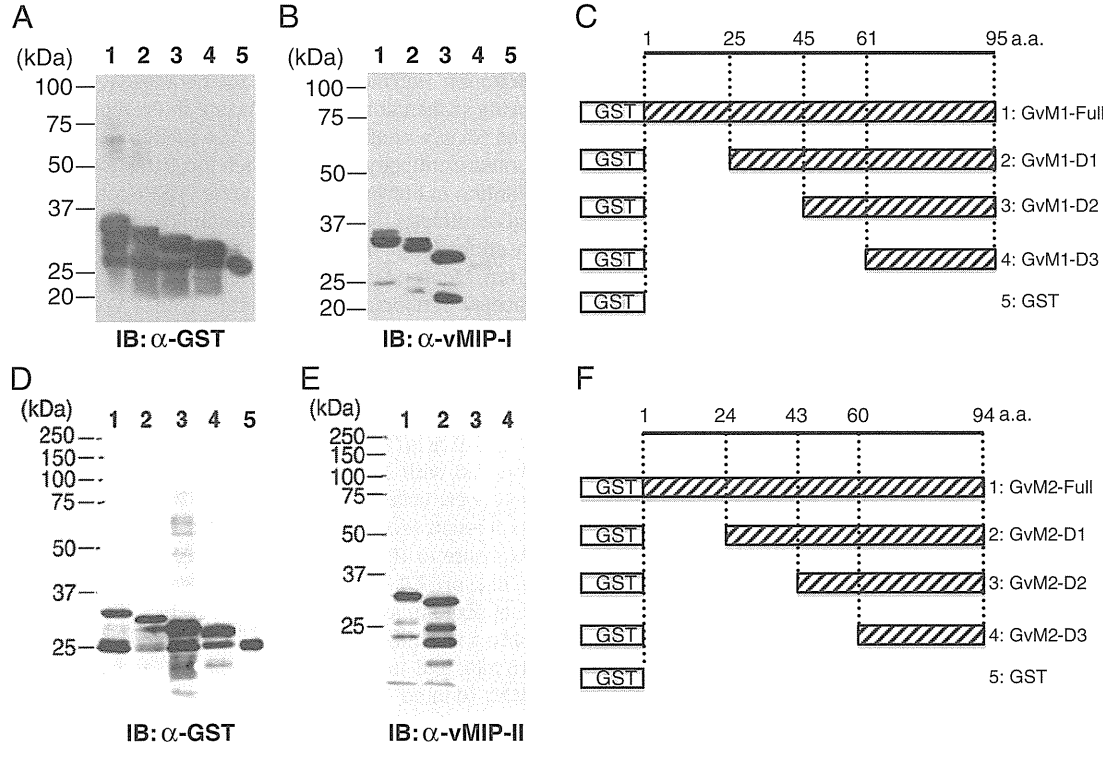

Fig. 2. Epitope mapping of the anti-vMIP-I and the anti-vMIP-II MAbs. To map the regions of vMIP-I and vMIP-II recognized by the anti-vMIP-I and anti-vMIP-II antibody, a series of GST-vMIP-I and GST-vMIP-II fusion proteins containing the individual regions of vMIP-I and vMIP-II were constructed as described in Fig. 2C and F, and the proteins were expressed in *E. coli*. The lysates of the fusion proteins, vMIP-I and vMIP-II, and its deletion mutants were immunoblotted with an anti-GST antibody (A and D) and an anti-vMIP-I (B) and an anti-vMIP-II antibody (E) to detect GST-vMIP-I or GST-vMIP-II fusion proteins. Lane 1, GvM1-Full; lane 2, GvM1-D1; lane 3, GvM1-D2; lane 4, GvM1-D3; lane 5, GvM1-D4; lan3 6, GST in Fig. 2A and B. Lane 1, GvM2-Full; lane 2, GvM2-D1; lane 3, GvM2-D2; lane 4, GvM2-D3; lane 5, GST (in D only) in Fig. 2D and E. Summary of GST-vMIP-I (C) and GST-vMIP-II (F) deletion mutants. Individual regions of vMIP-I and vMIP-II were cloned in-frame into the pGEX-5X-1 vector to generate GST-vMIP-I and GST-vMIP-II fusion proteins, respectively. The boxes at left indicate GST, and the white boxes with slashed lines indicate individual domains of vMIP-I and vMIP-II. 1, GvM1-Full(1-95a.a.); 2, GvM1-D1(25-95a.a.); 3, GvM1-D2(45-95a.a.); 4, GvM1-D3(61-95a.a.) in Fig. 2C, and 1, GvM2-Full(1-94a.a.); 2, GvM2-D1(24-94a.a.); 3, GvM2-D2(43-94a.a.); 4, GvM2-D3(60-94a.a.) in Fig. 2F.

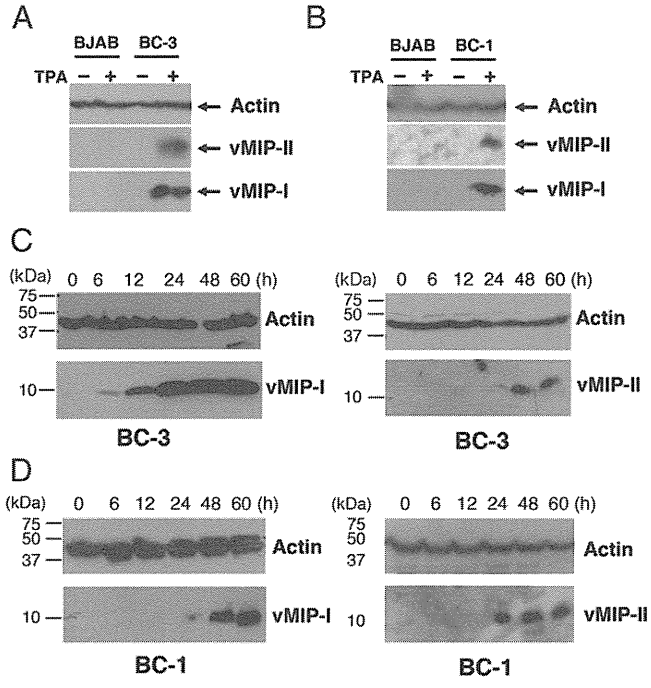

Fig. 3. Detection of vMIP-I and vMIP-II gene products in a KSHV-infected PEL cell line. BC-1 and BC-3 cells were treated with TPA for the indicated number of hours, and the whole-cell extract was prepared after the indicated time post-induction. vMIP-I and vMIP-II were detected by Western blotting and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

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

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

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

Transfection analysis of vMIP-I and vMIP-II

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

Antibodies and Western blotting

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

IFA

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