以下に，生物製剤の非臨床試験の実施において議論となることの多いもののうち，がん原性試験と生殖発生毒性試験の考え方について各論を記載する。

4.3 生物製剤のがん原性

生物製剤における過剰な薬理作用を介したがん原性の評価に際しては，薬理学的に適切な動物種を選択する必要がある。げっ歯類を用いることができる場合には，2年間の長期試験が実施可能であるが，中和抗体の産生により適切に評価できないことがある。また，サルのみで反応性がある場合やチンパンジーを除き適切な動物種が存在しない場合には，生涯投与試験は非現実的であり，動物福祉の観点で試験の実施は不可能である。そこで，適切な動物種が存在する場合，まずは慢性毒性試験において増殖性病変の有無を確認することが重要である。ただし，増殖性病変が見られなかったからといって，プロモーター作用の懸念がないとは結論できず，何らかの追加検討を考慮する必要があるかもしれない。そこで，標的細胞の増殖刺激に関するアプローチとして，慢性毒性試験における細胞増殖性（PCNA 免疫組織化学的染色等）の検討，複製 DNA 合成の検討，ヒトの培養細胞あるいは標的分子を発現した細胞を用いた増殖能の検討などが考慮される。一方，適切な動物種が存在しない場合，通常の毒性試験ではなく，相同タンパクあるいは相同抗体（surrogate antibody）を用いた反復投与毒性試験，ヒト化動物を用いた反復投与毒性試験，あるいは遺伝子改変動物を用いた自然発生腫瘍の検討等が考えられる。なお，一般に，生物製剤の類似物質に関する臨床データが十分に存在する場合には，動物実験での結果が発癌リスク評価に有用な追加情報とは考えにくい。

一般に，蛋白製剤やペプチドは細胞膜を通過せず，アミノ酸に分解されるため，DNA に作用することはないと考えられている。したがって，直接的な発癌作用を考慮する必要はない。ただし，バイオコンジュゲートの場合には，オーガニックリンカーの評価を考慮する必要がある。直接的な発癌作用はないとはいえ，いくつかの生物製剤は，非遺伝子障害性の発癌作用（プロモーター作用）を示すことが知られている。この原因は，過剰な薬理作用を介した腫瘍の誘発であり，成長促進作用を有する医薬品（成長因子，ホルモン，作動性モノクローナル抗体など）あるいは免疫抑制作用を有する医薬品（モノクローナル抗体）で認められているものがある。例として，メカニズムは明らかになっていないが，カルシトニンの長期投与によるげっ歯類での下垂体腫瘍，発生上皮小体ホルモンの長期投与（過剰な薬理作用）によるげっ歯類での骨肉腫の発生がある。なお，ラットおよびマウスの成長ホルモンの2年間の癌原性試験においては，陰性の結果が報告されている。

インスリン類縁体，分化因子および成長ホルモンのように分裂促進作用（発がんプロモーター）があることが示唆されている品目については，細胞分裂促進作用（mitogenicity）を *in vitro* で，

がん原性を *in vivo* で評価することが推奨される。*In vitro* の試験には陽性対照を使用し，*in vivo* では，通常は2年間のげっ歯類を用いたがん原性試験，細胞分裂作用が弱い試験物であれば，6ヶ月での評価で可能である。また，*in vivo* の評価として，ヒト由来腫瘍細胞をヌードマウスに移植したモデルにおいて，薬剤による腫瘍の成長（増殖促進や転移）を評価した事例があるようである。一方，免疫抑制剤は，げっ歯類で薬理学的活性を示さず，現時点では合意された癌原性の評価方法はない。抗IL-1受容体拮抗薬の場合，遺伝毒性はなく，ラット6ヶ月反復投与毒性試験で腫瘍や細胞増殖作用は認められていないにも関わらず，添付文書では悪性腫瘍に対する影響は不明であると記載されている。また，薬理作用を示さないという理由でげっ歯類の2年間のがん原性試験は実施されていないという事例もある。げっ歯類のがん原性試験が実施されていなくても，添付文書で発癌に関するリスクが記載されることになる。

また，多くの免疫抑制剤では，短期間の内にリンパ組織の増生を引き起こすため，直接的発癌性よりもプロモーター作用があると考えられている。この場合，2年間の癌原性試験がなくてもリスクの評価は可能と考えられるが，免疫抑制の強さは発癌を考慮すべき重要な要因であり，データがない場合であっても発癌の可能性のあることを添付文書に表示すること等を考慮する必要がある。

細胞医薬品の造腫瘍性については，FDAの考え方の推移について，“Points to consider in the characterization of cell lines used to produce biologicals”（1993年）において，athymicヌードマウスの皮下に細胞を移植し，3ヶ月間腫瘍ができるかということを評価するように記載されているが，その後の議論で，最近はこのPTCに書かれている方法を改良した試験法が良いと考えられている。さらに，2006年の“Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases”ガイドラインにて，基本的には同様の試験方法が書かれている。

以上のように，生物製剤の発癌性の評価は，薬理作用，反復毒性や患者集団特性等を考慮した上で，標準的な試験法よりも代替法が有用な場合が多いこと，2年間のがん原性試験は必ずしも必要でないことを考慮しなければならない。通常の新規化合物と生物製剤には安全性評価に必要となる毒性試験に明確な差があるわけで，ケースバイケースでの対応が必要である。

4.4　生物製剤の生殖・発生毒性試験

生物製剤について生殖・発生毒性試験が必要かは，剤型，薬理・生物活性，臨床適応，対象患者によって判断される。

特に持続的な免疫作用を有するモノクローナル抗体では，新生児の免疫機能も評価できるよう

に生殖・発生毒性試験の計画を設定することで，発育に及ぼす免疫毒性の可能性が検討できるかもしれない。一般に，生物製剤の類似物質に関する生殖・発生毒性試験のデータが十分に存在する場合には，適切な動物種がヒト以外の動物種で存在せず，かつ，作用機序から既知の医薬品の情報の利用が十分に使用可能という条件のもと，生殖・発生毒性試験は不要の場合もある。

毒性を正当に評価できる動物種がサルのみという場合，生物製剤における生殖・発生毒性試験の留意点について，サルでの生殖・発生毒性試験を実施する科学的根拠があるか，得られたデータが十分に意義のあるものであるかを考慮する必要がある。また，サルに代わる試験系として，遺伝子改変動物や相同タンパクを使ったげっ歯類での評価の可能性についての検討も必要である。生物製剤の生殖・発生毒性試験においては，通常使用されるげっ歯類やウサギでは中和抗体が産生され，試験系として不適切な場合がある。このような場合，その他の動物種としてサル（カニクイザル，アカゲザル）やハムスター等を用いた試験を考慮することになろう。現実的には，サルを用いた生殖・発生毒性試験が実施できる施設は世界的に見ても限られ，蓄積されているデータもまだ少ない。近年，いくつかの抗体医薬でサル生殖・発生毒性試験の実施があり，また，相同タンパクを用いたマウス生殖・発生毒性試験の報告もある。

生物製剤の生殖・発生毒性評価においては，科学的根拠にもとづいて，それぞれに適した試験系の選択にケースバイケースの考え方が重要である。さらに，新規の試験系を用いる場合には，科学的根拠や背景データの蓄積が極めて重要である。行政当局との相談を密接に行うことが望まれよう。

5. 生物製剤の承認申請

5.1 生物製剤の承認申請の考え方

前述のように，生物製剤には様々な剤型，臨床適応があり，承認申請の詳細をここで説明することは困難である。すでに承認申請のある蛋白製剤や抗体医薬については，FDA より提示されている Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use.（1997；http://www.fda.gov/cber/gdlns/ptc_mab.pdf）や，Draft guidance: Development of parathyroid hormone for the prevention and treatment of osteoporosis.（2000；http://www.fda.gov/cder/guidance/3789dft.htm）のようなガイドラインがあるので，参照されたい。細胞医薬などの場合には，近い将来承認事例も出てくることが予想されるので，その動向が注目されるところである。重要なことは，前臨床の段階（pre-IND フェーズ），早期臨床試験の段階（IND フェーズ），End-of Phase 2 ミーティングを経てフェーズ 3 臨床試験の段階と FDA 当局と積極的にかつ緊密に連携し，よくディスカッションをしながら開発を進めることである。承認申請に当

たっては，とくにEnd-of Phase 2ミーティングにおいて，フェーズ3臨床試験における臨床のゴールを設定するSpecial Protocol Assessment（SPA）にも真摯に対応し，同時に承認申請時のcommon technical document（CTD）に必要なデータを整理，明確化するべきである。常に忘れてはならないのは，行政側とてあたらしい生物製剤の評価についてはチャレンジも多く，開発者と二人三脚で新しい医療を切り開いていこうとする意識は高いということである。

本項目の最後に，現在欧米でトピックとなっている，ヒト型抗体医薬の臨床試験で有害事象があった際の市販後の考え方（RiskMAP）について紹介する。

5.2　生物製剤の承認とRiskMAP

先端医学の知見の進歩により新規メカニズムを有する医薬品の開発機会が増した。しかし，効果も高いが副作用の存在する医薬品も増加している。市販前の臨床試験ではわからなかった有害事象が医薬品の承認後に明らかになり，市販に際して通常以上の安全性の監視と活動が必要となる例も今後増えると考えられている。

抗体医薬natalizumabは，多発性硬化症やクローン病の治療薬として開発された・4インテグリンをターゲットとしたヒト化モノクローナル抗体である。無作為二重盲験試験においてnatalizumab投与群で新規炎症病変の発症は顕著に抑制されたことが認められ，2004年11月にFDAは，再発寛解した多発性硬化症に対してnatalizumabを承認した（商品名はTysabri, Biogen Idec社およびElan社から発売）。しかしながら，natalizumabを使用した多発性硬化症およびクローン病の臨床試験に参加した3人の患者において，進行性多病巣性白質脳障害（Progressive Multifocal Leukoencephalopathy；PML）が発症していたことが明らかになり，2005年2月には臨床試験及び販売が中止となった（その後3人のうち2人は死亡した）。PML発症のメカニズムは未だ明らかになっていない。

natalizumabの販売の再開について，関係学会や企業，有識者，FDAで大きな議論となり，その結果，2006年6月，FDAの医薬品安全部（Office of Drug Safety）はリスク最小化活動計画（Risk Minimization Action Plan；RiskMAP）というガイドラインの提示と，RiskMAPに従ったnatalizumabの適正使用を企業および医療機関に指示した。これによってnatalizumabはついに事実上の上市がなされることになった。RiskMAPとは，医薬品の使用にあたっての重要な特定されたリスク，重要な潜在的リスク，重要な不足情報を勘案し，通常の医薬品安全性監視以上にさらなる対応を義務付けるものであり，ベネフィットも高いがリスクも存在する医薬品に対して提示された考え方である。natalizumabの販売と使用にあたっては，とくにTysabri Outreach Unified Commitment to Health（TOUCH）プログラムが開始し，TOUCHプログラムに参加する施設，患者に限って処方されること，PMLの発症を早期に診断するために，投薬前の患者に

MRI 撮像を行うこと，また初回投与後，定期的に患者の評価結果を販売企業に報告することなどが義務付けられることとなった。

以上のように，昨今，医薬品の潜在的リスクも複雑化していることは否めない。このため，研究開発のフェーズにおける安全性評価の際には，承認申請時に，市販後安全性評価も見据えた対応が必要となりつつあるのである。

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Loop-Mediated Isothermal Amplification-Based Diagnostic Assay for Monkeypox Virus Infections

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Monkeypox virus (MPXV) causes a smallpox-like disease in non-human primates and humans. This infection is endemic to central and western Africa. MPXV is divided into two genetically different groups, Congo Basin and West African MPXV, with the former being the more virulent. A real-time quantitative MPXV genome amplification system was developed for the diagnosis of MPXV infections using loop-mediated isothermal amplification (LAMP) technology. Primers used for genome amplification of Congo Basin (C-LAMP), West African (W-LAMP), and both Congo Basin and West African (COM-LAMP) MPXV by LAMP were designed according to the nucleotide sequences of the Congo Basin-specific D14L gene, the West African-specific partial ATI gene, and the partial ATI gene that is shared by both groups, respectively. The sensitivity and specificity of the LAMP were evaluated with nested PCR using peripheral blood and throat swab specimens collected from Congo Basin MPXV or West African MPXV-infected monkeys. The sensitivity and specificity of COM-LAMP, C-LAMP, and W-LAMP were 80% (45/56) and 100% (64/64); 79% (19/24) and 100% (24/24); and 72% (23/32) and 100% (40/40), respectively. The viremia level determined by LAMP assays increased with increases in the severity of the monkeypox-associated symptoms. The newly developed LAMP assay was confirmed to be a rapid, quantifiable, and highly sensitive and specific system effective in the diagnosis of MPXV infections. The LAMP assays made it possible to discriminate between Congo Basin and West African MPXV. The LAMP developed in this study is useful not only for diagnosis of but also for the assessment of MPXV infections. ***J. Med. Virol. 81:1102–1108, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: loop-mediated isothermal amplification; LAMP; monkeypox; monkeypox virus; diagnosis

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INTRODUCTION

Monkeypox virus (MPXV) belongs to the genus *orthopoxvirus* in family *Poxviridae*, and, together with variola virus, is a causative agent for smallpox. MPXV was first isolated in 1958 from cynomolgus monkeys with symptoms similar to those of smallpox in humans [von Magnus et al., 1959]. It was identified that MPXV causes a smallpox-like disease in humans (human monkeypox) in 1970 [Ladnyj et al., 1972]. The disease is endemic to the rainforests of central and western Africa, where some species of ground squirrels, which are suggested to be the reservoir, are prevalent [Meyer et al., 2002]. People living in the endemic regions still suffer from this infection. A sporadic outbreak of human monkeypox occurred in the USA in 2003 [Likos et al., 2005]. This was the first outbreak of human monkeypox outside Africa. The source of the outbreak was MPXV-infected ground squirrels, such as African dormice and Gambian giant rats, imported from western Africa through Accra, Ghana. This event indicates that there is the possibility that MPXV could spread outside Africa and cause human monkeypox even in countries currently free from this infection. Furthermore, MPXV is categorized as an important bio-weapons. MPXV is divided into two genetic distinct groups, Congo Basin and West African MPXV. Congo Basin MPXV was

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reported to be more virulent than West African MPXV [Chen et al., 2005]. Therefore, there is a need to develop rapid and accurate diagnostic systems with which to discriminate between Congo Basin and West African MPXV.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that works by autocycling strand displacement DNA synthesis by *Bst* DNA polymerase, and has been confirmed to be simple to use [Notomi et al., 2000]. The amplification is conducted under isothermal conditions ranging from 60 to 65°C with DNA polymerase and, usually, four primers recognizing six distinct target regions (4-primer-based LAMP), making this assay highly specific. If two additional "loop primers" are included in the LAMP assay, the reaction time can be reduced (6-primer-based LAMP) [Nagamine et al., 2002]. Recently, the LAMP-based diagnosis for several virus infections have been developed [Hong et al., 2004; Kaneko et al., 2005; Imai et al., 2006; Parida et al., 2006, 2007; Suzuki et al., 2006; Shirato et al., 2007].

In the present study, 6-primer-based LAMP was applied for the development of diagnostic systems for MPXV infections. Three LAMP assays, which detected specifically Congo Basin and West African MPXV, Congo Basin but not West African MPXV, and West African but not Congo Basin MPXV, respectively, were developed. The usefulness of the LAMP assays for the diagnosis and assessment of MPXV infections was evaluated using peripheral blood and throat swab specimens collected from MPXV-infected monkeys.

MATERIALS AND METHODS

Viruses and Cells

Congo Basin MPXV (Zr-599, Congo-8, and V97-I-008 strains), West African MPXV (Sierra Leone, Liberia, Copenhagen, and Anteatan strains), cowpox virus (Brighton Red strain), camelpox virus (J1 strain), ectromelia virus (Hamstead strain), and vaccinia virus (Lister strain) stored at the National Institute of Infectious Diseases, Tokyo, Japan (NIID), were used. The MPXV strains and other orthopoxviruses were grown on Vero and HeLa cells, respectively.

Extraction and Purification of Virus Genome

Viral DNA was extracted from MPXV (Zr-599)-, MPXV (Liberia)-, cowpox virus-, camelpox virus-, ectromelia virus-, or vaccinia virus-infected cells using the Hirt extraction method [Hirt, 1967]. Viral DNA in the peripheral blood and throat swab specimens was purified using a Viral Nucleic Acid Purification Kit™ (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and stored at −30°C until use.

Real-Time Quantitative PCR for Orthopoxvirus

A SYBR Green-based real-time quantitative PCR (pox-qPCR) was developed. Primers, forward primer [H2Rf (5′-CGGTTAACGATTGGAAATCATTAACGG-3′)] and reverse primer [H2Rr (5′-CCTCGCCTAA-TAGCTTGCG-3′)], used in the pox-qPCR were designed according to the nucleotide sequences of the *H2R* gene shared by viruses in the genus *Orthopoxvirus*. Standard DNA, a pGEM-T easy vector (GH Health Care Japan, Tokyo, Japan) inserted with the partial *H2R* gene amplified in PCR with the above primer set (H2f and H2r), was used for the determination of the copy number of the virus genome of MPXV, cowpox virus, camelpox virus, ectromelia virus, and vaccinia virus. The reaction conditions were as follow: one cycle of 95°C for 10 min for denaturation, 45 cycles of 95°C for 15 sec, 63°C for 5 sec, 72°C for 10 sec, followed by one cycle of 73°C for 15 sec. PCR amplification was performed using a LightCycler FastStart DNA Master SYBR Green I™ kit (Roche Diagnostics Ltd.) in a 20 µl-volume format containing 5 µl of template DNA according to the manufacturer's instructions.

LAMP

The 6-primer-based LAMP consists of six primers; two outer primers (F3 and B3), a forward inner primer (FIP), a backward inner primer (BIP), and two loop primers (LF and LB) [Nagamine et al., 2002]. Three LAMP assays were developed for the amplification of the genomes of both Congo Basin and West African MPXV (COM-LAMP), of the genomes of Congo Basin but not West African MPXV (C-LAMP), and of the genomes of West African but not Congo Basin MPXV (W-LAMP). The primers for COM-LAMP, C-LAMP, and W-LAMP were designed according to the nucleotide sequences of the A-type inclusion body (ATI) shared by both Congo Basin and West African MPXV, those of the Congo Basin MPXV-specific *D14L* gene [Likos et al., 2005], and those of the West African MPXV-specific partial ATI gene [Saijo et al., 2008]. The primer Loop-B-COM in the COM-LAMP was designed to anneal the region containing a deletion of eight nucleotide residues observed only in MPXV but not in other orthpoxviruses [Neubauer et al., 1998]. The nucleotide sequences of the primers used in each LAMP assay are shown in Table I. The LAMP reaction was performed with a Loopamp DNA Amplification kit™ (Eiken Chemical Co., Ltd., Tochigi, Japan). The reaction mixture (25 µl) containing 40 pmol of each inner primer, FIP and BIP, 5 pmol of each outer primer, F3 and B3, and 20 pmol of each loop primer, LF and LB, 2 times concentrated reaction mix (12.5 µl), *Bst* polymerase (1.0 µl), and 2.0 µl of sample was incubated at 63°C with a Loopamp real-time turbidmeter (LA-200; Teramecs, Tokyo, Japan) for 1 hr, followed by incubation at 80°C for 2 min to terminate the reaction. To confirm whether the LAMP amplification products were authentic, they were digested with a designated restriction enzyme and electrophoresed in a 3% agarose gel containing ethidium bromide for separation. The DNA fragments were then visualized. The amplified COM-LAMP and C-LAMP products were digested with *Taq*I, and the W-LAMP product was digested with *Bgl*II.

TABLE I. Names and Sequences of Primers for COM-LAMP, C-LAMP, W-LAMP

Assay	Target gene	Primers	
		Name	Nucleotide sequence
COM-LAMP	ATI	FIP-COM	5′-TGGAGTCTGCTAATCTCTGTAAGATTAGAGAACTAGAGAATAAGTTGACC-3′
		F3-COM	5′-CACAAGAAGTTGATGCACTG -3′
		BIP-COM	5′-TGAGTGAATGCCGTGGAAATGCGCAGTCGTTCAACTGTA-3′
		B3-COM	5′-CAGCATTGATTTCATTATTACGT-3′
		Loop-F-COM	5′-CGCTCTCGATGCAGTC-3′
		Loop-B-COM	5′-CAGAGATTACAATCTAGAATCTCAG-3′
C-LAMP	D14L	FIP-C	5′-TGGGAGCATTGTAACTTATAGTTGCCCTCCTGAACACATGACA-3′
		F3-C	5′-TGGGTGGATTGGACCATT-3′
		BIP-C	5′-ATCCTCGTATCCGTTATGTCTTCCCACCTATTTGCGAATCTGTT-3′
		B3-C	5′-ATGGTATGGAATCCTGAGG-3′
		Loop-F-C	5′-GATATTCGTTGATTGGTAACTCTGG-3′
		Loop-B-C	5′-GTTGGATATAGATGGAGGTGATTGG-3′
W-LAMP	ATI	FIP-W	5′-CCGTTACCGTTTTTACAATCGTTAATCAATGCTGATATGGAAAAGAGA-3′
		F3-W	5′-TACAGTTGAACGACTGCG-3′
		BIP-W	5′-ATAGGCTAAAGACTAGAATCAGGGATTCTGATTCATCCTTTGAGAAG-3′
		B3-W	5′-AGTTCAGTTTTATATGCCGAAT-3′
		Loop-F-W	5′-GATGTCTATCAAGATCCATGATTCT-3′
		Loop-B-W	5′-TCTTGAACGATCGCTAGAGA-3′

Standard DNA for Quantification in Each LAMP Assay

Standard DNA for the determination of the copy number of MPXV DNA was the pGEM-T easy vector inserted with the partial ATI gene amplified using the primer set, ATI-up-1 and ATI-low-1, for COM-LAMP and W-LAMP [Meyer et al., 1994]. The standard DNA for C-LAMP was the pGEM-T easy vector inserted with the partial *D14L* gene amplified using the primer set D14L-F (5′-GTTGTATGAGAGTATGATC-3′) and D14L-R (5′-TATGAAGGTGGAGAGCGTGAC-3′).

Nested PCR

A nested PCR, with which very low copies of the MPXV genomes could be amplified, was developed. The first round PCR was carried out using the primer set Gabon-1 (5′-GAGAGAATCTCTTGATAT-3′) and Gabon-2 (5′-ATTCTAGATTGTAATC-3′) [Neubauer et al., 1998]. The primers for the second round PCR were designed as follows: forward primer nest-Fa1 (5′-GCACACGCAATCAAGAAGAC-3′) and reverse primer nest-Ra1 (5′-ATTGTAATCTCTGTAGCATTTC-3′) to amplify the inner region of the product in the first round PCR. The reaction conditions were as follow: one cycle of 94°C for 5 min for denaturation, followed by 25 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, followed by one cycle of 72°C for 5 min. The PCR were performed using the GeneAmp PCR system 9700 (Applied Biosystems, Carlsbad, CA) with the Expand High Fidelity System™ (Roche Diagnostics Ltd.) according to the manufacturer's instructions.

Monkeys and MPXV-Challenge Experiments

Thirteen cynomolgus monkeys (*Macaca fascicularis*) born and raised at the Tsukuba Primate Center for Medical Science, National Institute of Biomedical Innovation, Tsukuba, Japan, were used. The monkeys were infected with MPXV in a highly contained laboratory, in which glove-box type safety cabinet was installed, at the NIID, Tokyo, Japan. All monkeys were infected with MPXV (Zr-599 or Liberia strain) at a dose of 10^6 plaque forming units. Smallpox vaccine, LC16 m8 and Lister, was confirmed to be effective in protecting the monkeys from generalized monkeypox [Saijo et al., 2006]. To demonstrate the various levels of the clinical manifestations of monkeypox, monkeys were immunized with LC16m8 or Lister for a designated period before MPXV challenge as shown in Table II. One monkey was infected with Zr-599 followed by immunization with LC16 m8. Blood samples and throat swab specimens were collected every 3 or 4 days after challenge. Clinical manifestations, such as changes in body weight, volume of food and water consumed, skin lesions, and the appearance of feces, were observed daily. The skin surface was observed carefully, and body temperature and weight were measured every 3–4 days while the monkeys were anesthetized.

Quantitative Real-Time PCR for MPXV

MPXV genome copies in the peripheral blood and throat swab specimens were determined by real-time quantitative PCR (qPCR) as reported previously [Saijo et al., 2006].

Statistical Analysis

Sensitivity and specificity were defined as the probability that the LAMP assay result was positive when the nested PCR showed a positive result, and as the probability that the LAMP assay result was negative when the nested PCR showed a negative result, respectively. The correlation coefficient(r) between the viremia levels detected by COM-LAMP and by qPCR was obtained using Pearson's correlation coefficient.

TABLE II. Experimental Data and Clinical Manifestations of Monkeypox for Monkey Subjects Used in This Study

ID	Virus inoculated	Route	Vaccination	Days from vaccination to challenge	No. of clinical samples tested		No. of papulovesicular lesions	Severity	Outcome
					PBC	TS			
Z-01-SC	Zr-599	SC	—	—	5	5	388	Severe	Sacrificed
Z-02-SC		SC	LC16m8	0	7	7	691	Severe	Survived
Z-03-SC		SC	LC16m8	3	6	6	286	Mild	Survived
Z-04-SC		SC	LC16m8	7	6	6	0	Asymptomatic	Survived
L-01-SC	Liberia	SC	—	—	7	7	196	Moderately severe	Survived
L-02-SC		SC	—	—	4	0	—	Severe	Sacrificed
L-03-SC		SC	—	—	7	0	29	Moderately severe	Survived
LC-04-IN		IN	—	—	8	0	10	Mild	Survived
LC-05-IN		IN	—	—	8	0	16	Mild	Survived
LC-06-IN		IN	Lister	42	8	0	0	Asymptomatic	Survived
LC-07-IN		IN	Lister	42	9	0	0	Asymptomatic	Survived
LC-08-IN		IN	Lister	42	7	0	0	Asymptomatic	Survived
LC-09-IN		IN	LC16m8	42	7	0	0	Asymptomatic	Survived

TS, throat swab specimens; PBC, peripheral blood cells; SC, subcutaneous inoculations; IN, intranasal inoculations.

RESULTS

Detection of Orthopoxviruses by COM-LAMP

At least 10^2 copies of MPXV Zr-599 and Liberia genomes were detected by the COM-LAMP assay. 10^6 copies of camelpox virus genomes, but only 10^5 copies of the virus genome, were positive by the COM-LAMP assay (Table III). However, the amplified DNA from the MPXV genomes could be differentiated from that from camelpox virus by restriction enzyme treatment (Fig. 1).

Detection of the Genomes of Congo Basin and West African MPXV by C-LAMP and W-LAMP

MPXV Zr-599 was detected by C-LAMP, but not by W-LAMP. On the other hand, MPXV Liberia was detected by W-LAMP but not by C-LAMP (Table III). All the Congo Basin MPXV genomes but not the West African MPXV genomes were detected by C-LAMP. On the other hand, all the West African MPXV genomes but not the Congo Basin MPXV genomes were detected by W-LAMP (data not shown).

Detection Limit of COM-LAMP, C-LAMP, and W-LAMP

The detection limits of COM-LAMP, C-LAMP, and W-LAMP were approximately $10^{2.0}$, $10^{2.4}$, 10^3 copies/reaction of standard DNA, respectively. These values were calculated from the results obtained from 9 independently repeated experiments.

Sensitivity and Specificity of the LAMP Assays

Detection of MPXV genomes in clinical samples, peripheral blood cells and throat swab specimens, was tested by each of the LAMP assays and nested PCR. The sensitivity and specificity of COM-LAMP, C-LAMP, and W-LAMP were 80% (45/56) and 100% (64/64); 79% (19/24) and 100% (24/24); and 70% (23/32) and 100% (40/40), respectively (Table IV).

Relationship Between Severity of Symptoms and Viremia Level as Determined by COM-LAMP

The severity of monkeypox symptoms observed during the challenge experiments is shown in Table II. The

TABLE III. The Reactivity of the Tested Orthopoxvirus DNAs in COM-LAMP and of MPXV DNAs in C-LAMP and W-LAMP

LAMP	Virus	Virus genome (copies/reaction)					
		10^1	10^2	10^3	10^4	10^5	10^6
COM-LAMP	MPXV Zr-599	−	+	+	+	+	+
	MPXV Liberia	−	+	+	+	+	+
	Ectromelia	NT	NT	NT	−	−	−
	Cowpox	NT	NT	NT	−	−	−
	Camelpox	NT	NT	NT	−	−	+
	Vaccinia	NT	NT	NT	−	−	−
C-LAMP	MPXV Zr-599	−	−	+	+	+	+
	MPXV Liberia	−	−	−	−	−	−
W-LAMP	MPXV Zr-599	−	−	−	−	−	−
	MPXV Liberia	−	+	+	+	+	+

NT, not tested.

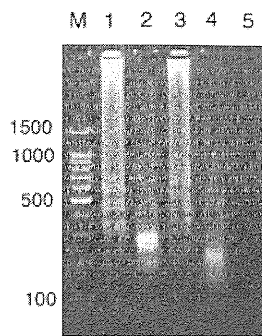

Fig. 1. DNA products from MPXV and camelpox virus amplified with COM-LAMP and separated in a 3% agarose gel by electrophoresis (**lanes 1** and **3**, respectively). To discriminate MPXV from camelpox, MPXV-LAMP and camelpox virus-LAMP products were treated with a restriction enzyme (*Taq*I) (**lanes 2** and **4**, respectively). A 100 bp-DNA ladder marker and negative control are also shown (**lanes M** and **5**, respectively).

infection was lethal in one naive monkey (Z-01-SC). The viremia level determined by the COM-LAMP assay continued to increase until sacrifice. The symptoms in the monkey pre-immunized 3 days before challenge (Z-03-SC) were less severe than those of the post-exposure vaccinated monkey (Z-02-SC). The viremia level in monkey (Z-02-SC) was significantly higher than that in monkey (Z-03-SC) throughout the observation period. The monkey pre-immunized 7 days before challenge (Z-04-SC) showed an asymptomatic infection. No viremia was demonstrated in this subject by the COM-LAMP assay (Fig. 2A). The severer the level of monkeypox-associated symptoms observed, the higher the viremia level determined by COM-LAMP. A similar phenomenon was observed in experiments in which monkeys were infected with MPXV Liberia through intranasal inoculation or the subcutaneous route at a dose of 10^6 PFU (Fig. 2B). The monkeypox symptoms in one monkey (L-03-SC) were so severe that the subject was sacrificed due to ethical considerations. The two monkeys (L-01-SC and L-020SC) infected with MPXV Liberia through the subcutaneous route showed moderately severe symptoms and survived. The two monkeys (L-04-IN and L-05-IN) showed mild symptoms with less than 20 papulovesicular skin lesions. Furthermore, viremia was demonstrated on Day 3 in the subcutaneously infected monkeys but not in the intranasally infected subjects.

Relationship Between Virus Loads as Determined by COM-LAMP and qPCR

The relationship between the virus load in the clinical samples determined by COM-LAMP and those determined by qPCR is shown in Figure 3. The correlation coefficient was 0.60, which represents a strong positive correlation.

DISCUSSION

Nucleic acid amplification-based diagnostic assays have become a gold standard for the rapid diagnosis of viral infections. Several PCR assays, such as conventional PCR and real-time quantitative PCR, have been reported for MPXV [Ibrahim et al., 1997; Neubauer et al., 1998; Kulesh et al., 2004; Aitichou et al., 2005, 2008; Saijo et al., 2006, 2008; Scaramozzino et al., 2007]. The real-time quantitative PCR assays have the advantages of rapidity, quantification-capacity, detection in a real-time manner, and high sensitivity. However, these nucleic acid amplification methods require high-precision instruments such as LightCycler instruments (Roche Diagnostics Ltd.). On the other hand, LAMP can be carried out without using such instruments. Furthermore, virus genomes can be detected within a shorter time and in a real-time manner. If turbidity detection is performed using a Loopamp real-time turbidmeter (LA-200), the virus genomes can be detected in a real-time manner along with genome quantification.

Three LAMP assays, COM-LAMP, C-LAMP, W-LAMP, were developed in this study. Using these assays, it was possible to detect the genomes of the Congo Basin and West African MPXVs and to differentiate between the genomes of the Congo Basin and of West African MPXVs by a combination of the three LAMP assays.

10^6 copies/reaction of the camelpox virus genome showed a positive reaction in the COM-LAMP (Table III), indicating that a positive reaction in the COM-LAMP assay does not always indicate an MPXV infection. The homology of the Loop-B-COM with

TABLE IV. Relationship Between the Results Obtained From the Nested PCR and LAMP Assays

	Samples from Zr-599-challenged monkeys Nested PCR		Samples from Liberia-challenged monkeys Nested PCR	
LAMP method	Positive	Negative	Positive	Negative
COM-LAMP				
Positive	21	0	24	0
Negative	3	24	8	40
C-LAMP				
Positive	19	0	0	0
Negative	5	24	33	39
W-LAMP				
Positive	0	0	23	0
Negative	24	24	9	40

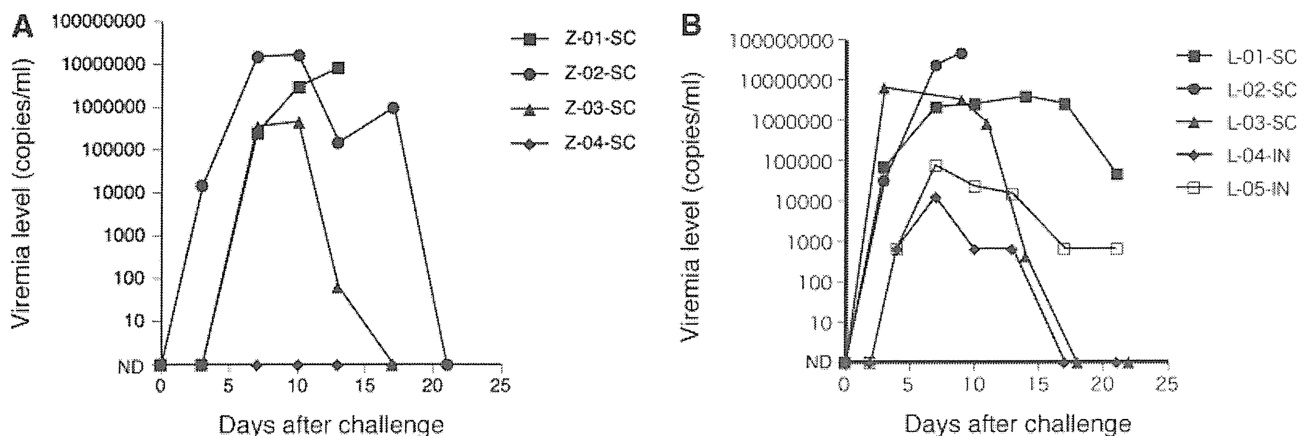

Fig. 2. Viremia level in peripheral blood collected from MPXV Zr-599-infected monkeys (**A**) and those in MPXV Liberia-infected monkeys (**B**) as determined by COM-LAMP. "ND" indicates it was below the detection level.

camelpox virus ATI-gene (80%) was higher than those with vaccinia virus and cowpox virus (76%), while there were no significant differences in homology of the other primers among these orthopoxviruses. Although further study is needed, the difference might be responsible for positive reaction of camelpox virus in COM-LAMP assay. Although a positive reaction in COM-LAMP does not indicate MPXV genome-positive, the amplified products of MPXV DNA and the other orthopoxvirus DNAs could be differentiated by restriction enzyme treatment or a combination of the COM-LAMP, C-LAMP and W-LAMP assays. Furthermore, when the viremia level determined by COM-LAMP was much less than that determined by C-LAMP, the samples can be understood to contain orthopoxviruses other than MPXV as orthopoxviruses such as camelpox and vaccinia viruses possesses a similar nucleotide sequence to the *D14L* gene of Congo Basin MPXV (data not shown). Although the data is not shown here, the genomes of herpes simplex virus and varicella zoster virus, which cause vesicular skin infections in humans and must be differentiated from human monkeypox, showed negative reactions in the newly developed LAMP assay. The corresponding genomes in variola virus, a causative agent for smallpox, to the partial ATI gene amplified by the COM-LAMP assay do not possess the *Taq*I restriction site, suggesting that the differentiation of MPXV from variola virus is possible by the COM-LAMP assay.

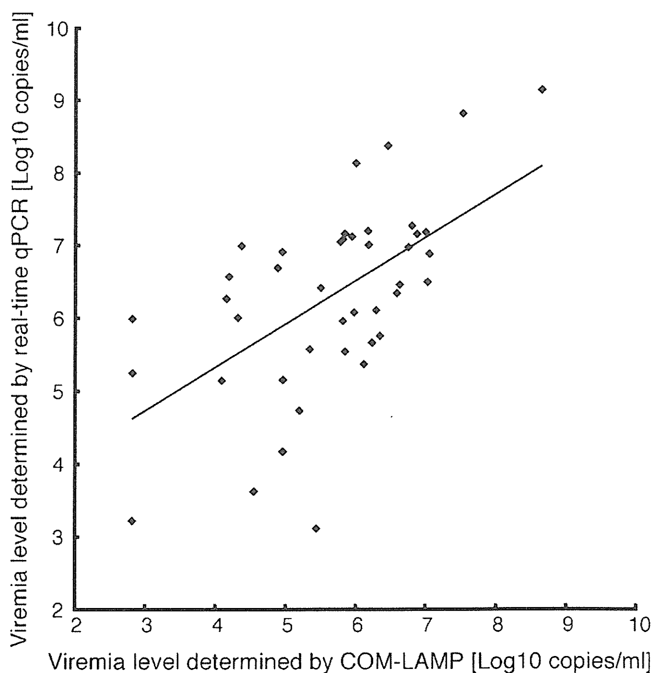

Fig. 3. Correlation between viral loads as determined by LAMP assays and real-time qPCR.

Three LAMP assays were evaluated in comparison with nested PCR. The sensitivity and specificity of the three LAMP assays when compared with the nested PCR were approximately 70–80% and 100%, respectively (Table IV). Because the nested PCR for the amplification of genome sequences is quite sensitive, the sensitivity of the LAMP assay was calculated to be 70–80% in this study. However, the sensitivity of the COM-LAMP assay was much higher when compared with the conventional PCR reported previously [Neubauer et al., 1998]. The viremia level determined by COM-LAMP was associated with the severity of clinical symptoms of monkeypox. The most significant advantages of LAMP assay over conventional PCR and real-time quantitative PCR is that the assay is simpler to perform. No highly specialized instruments are necessary for the LAMP assay, and even the detection of the turbidity derived from the accumulation of byproduct can be done visually [Mori et al., 2001]. It is concluded that the newly developed LAMP assays afford a valuable tool not only for the diagnosis of but also for the assessment of MPXV infections.

In summary, a sensitive, specific and rapid LAMP system for the detection of the MPXV genome was developed. Using this technology, MPXV can be differentiated into Congo Basin strains or West African

strains. This technology offers great benefits in the control of outbreaks of MPXV infections and in the assessment of the course of MPXV infections. Furthermore, the newly developed LAMP system may offer advantages in the diagnosis of human monkeypox, which would need to be differentiated from smallpox in the event of a variola virus-associated bioterrorism attack.

ACKNOWLEDGMENTS

The challenge experiments with MPXV were conducted in the high-containment laboratory at the NIID, Japan. All animal procedures were approved by the Committees on Biosafety and Animal Handling and Ethical Regulations of the National Institute of Infectious Diseases, Japan. Animal research was undertaken in compliance with the guidelines issued from the Ministry of Health, Labor and Welfare, "The Fundamental guidelines for proper conduct of animal experiment and related activities in institutions under jurisdiction (June 2006)." Our animal work also adhered to the principles stated in the guidelines.

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Short Communication

Virulence and pathophysiology of the Congo Basin and West African strains of monkeypox virus in non-human primates

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Monkeypox virus is divided into Congo Basin and West African strains. The virulence and pathophysiology of two strains, Zr-599 (a Congo Basin monkeypox virus) and Liberia (a West African monkeypox virus), were evaluated in non-human primates. Four monkeys were infected by the subcutaneous (SC) and two by the intranasal (IN) inoculation routes for Zr-599 and Liberia at a dose of 10^6 p.f.u. One monkey in the Liberia/SC group was demonstrated to be co-infected with Gram-positive cocci and was excluded from analyses. Infections in three of the four Zr-599/SC monkeys and in one of the three Liberia/SC monkeys were fatal. Virus genome levels in blood in the Zr-599/SC monkeys were approximately 10 times higher than those in the Liberia/SC monkeys. Zr-599 affected respiratory, genito-urinary and gastrointestinal tract organs more severely than Liberia. Zr-599 was more virulent than Liberia and one of the factors might be the difference in organ tropism.

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The species *Monkeypox virus* belongs to the genus *Orthopoxvirus*, family *Poxviridae*. Monkeypox viruses cause human monkeypox in humans inhabiting the rainforests of central and western Africa (Arita *et al.*, 1985; Damon, 2007; Heymann *et al.*, 1998). Wild rodents (*Funiscius anerythrus* and *Helioscirus rufobrachium*) were implicated as the most probable reservoir (Khodakevich *et al.*, 1987). Human monkeypox is endemic to central and western Africa (Khodakevich *et al.*, 1988). An outbreak of human monkeypox was reported in the Democratic Republic of Congo (DRC) (Khodakevich *et al.*, 1988; Learned *et al.*, 2005; Mukinda *et al.*, 1997). Human monkeypox outbreaks outside Africa were first reported in the USA in 2003 (Di Giulio & Eckburg, 2004; Guarner *et al.*, 2004; Reed *et al.*, 2004). In that outbreak, patients acquired the virus from prairie dogs (*Cynomys* spp.) that became ill after contact with various exotic rodents shipped from Ghana, Africa (Reed *et al.*, 2004).

Two clades of monkeypox virus exist: West African and Congo Basin monkeypox virus (Likos *et al.*, 2005). Human and monkey disease virulence differs between Congo Basin and West African strains, the former being more virulent in non-human primates (Chen *et al.*, 2005). The clinical manifestations of human monkeypox are reported to be similar to those of smallpox (Arita *et al.*, 1985; Breman *et al.*, 1977, 1980; Foster *et al.*, 1972; Janseghers *et al.*, 1984; Jezek & Khodakevich, 1987; Stagles *et al.*, 1985). This study describes the clinical manifestations and laboratory and pathological findings in cynomolgus monkeys infected with Congo Basin or West African monkeypox virus. The virulence of Congo Basin and West African monkeypox virus was compared. Furthermore, the pathophysiological mechanisms behind the difference in virulence between these two monkeypox viruses were elucidated.

Monkeypox virus strains Zr-599 (a representative Congo Basin strain) and Liberia (a representative West African strain) were used. Zr-599, isolated from a patient in the DRC, and Liberia, isolated from a patient with human monkeypox in Liberia, were assigned to the Congo Basin and the West African clades, respectively, according to A-type inclusion body gene sequence (Likos *et al.*, 2005). Virus solution for challenge experiments was prepared by disruption of Vero E6 cells infected with each monkeypox

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virus strain in a sonicator (TITEC Ultra S Homogenizer UP-5) for 30 s at full power, followed by high-speed centrifugation (3500 r.p.m. for 5 min at 4 °C). The infectious dose of the virus was determined by plaque assay.

Twelve cynomolgus monkeys (*Macaca fascicularis*) were used (see Supplementary Table S1, available in JGV Online). They were classified into four groups: Zr-599/SC, Liberia/SC, Zr-599/IN and Liberia/IN. Monkeys #4651 and #4653 (Zr-599/SC) and #4595 and #4596 (Liberia/IN) were used in a previous study in which the efficacy of a smallpox vaccine, LC16m8, was evaluated (Saijo *et al.*, 2006). Other monkeys were also used as control subjects in the study for the evaluation of LC16m8 efficacy. A monkey in the Liberia/SC group (#4567) died on day 10 post-inoculation. This subject was demonstrated to be co-infected with Gram-positive cocci and was excluded from further analyses.

Complete blood-cell counts in peripheral blood collected in sodium heparinized tubes were measured. C-reactive protein (CRP) was measured as an indicator of inflammation level.

Vaccinia virus-specific antibody levels were measured by ELISA using the entire suite of vaccinia virus proteins as antigens, as reported previously (Morikawa *et al.*, 2005; Saijo *et al.*, 2006). Although the IgG response in monkeys #4651 and #4653 from the Zr-599/SC group and #4595 and #4596 from the Liberia/IN group had already been determined as reported previously (Saijo *et al.*, 2006), the IgG response in all of the monkeys, including the four previously tested monkeys, was determined simultaneously. Virus genome levels were determined by a quantitative real-time PCR (qPCR) method as reported previously (Saijo *et al.*, 2006, 2008). Although the virus genome level in monkeys #4651 and #4653 from the Zr-599/SC group and #4595 and #4596 from the Liberia/IN group had already been determined in a previous study (Saijo *et al.*, 2006), the virus genome level in the peripheral total blood of all of the monkeys, including the four previously tested monkeys, was determined simultaneously. All challenge experiments were conducted in a highly contained laboratory in which a glovebox class III safety cabinet was installed. The monkeys were anaesthetized and inoculated intranasally (IN) with 0.5 ml virus solution containing 1×10^6 p.f.u. Zr-599 or Liberia by using an atomizer (Keytron Co.) to atomize the virus solution, or inoculated subcutaneously (SC) with 0.5 ml virus solution containing 1×10^6 p.f.u. Zr-599 or Liberia. After the challenge, blood was drawn every 2–4 days. Clinical manifestations, such as volume of food and water consumed, appearance of faeces, etc., were observed every day. The skin surface was observed carefully, and body (anal) temperature and mass were measured.

After sacrifice under anaesthesia, skin, lymphoreticular system structures (lymph nodes, spleen, thymus, pharynx and tonsils), gastrointestinal tract organs (including the liver and pancreas), genito-urinary tract organs (kidneys, bladder, testes, ovaries and uterus), endocrine organs (adrenal glands and thyroid), respiratory tract organs (trachea and lungs), the heart as the cardiovascular organ, and central nervous system (CNS) organs (brain and spinal cord) were excised, fixed in 10 % formalin in PBS and embedded in paraffin. They were then examined for micropathology and the presence of monkeypox virus antigens by immunohistochemical analyses as reported previously (Nagata *et al.*, 2001, 2002; Saijo *et al.*, 2006).

Infections in three of the four Zr-599/SC monkeys and one of the three Liberia/SC monkeys were fatal, whereas all Zr-599/IN and Liberia/IN monkeys survived (see Supplementary Table S1, available in JGV Online). In Zr-599/SC monkeys, body mass decreased sharply by 10–20 % after challenge without any sign of recovery except for one subject that survived, whereas body mass in Liberia/SC monkeys decreased less sharply. There was a tendency for body temperature to rise for the first week after virus inoculation in all groups (see Supplementary Fig. S1, available in JGV Online).

Papulovesicular rashes appeared on days 7–9 after monkeypox virus inoculation. The general condition of the monkeypox virus-infected monkeys deteriorated and their activity decreased from day 6 to day 11 post-inoculation. The mean number of papulovesicular lesions in the Zr-599-infected monkeys ($n=369$) was higher than that in the Liberia-infected monkeys ($n=226$) (Supplementary Table S1). Skin and gross lesions in the internal organs in a Zr-599-infected subject (#4653) and a Liberia-infected subject (#4625) on day 18 post-inoculation are shown in Fig. 1. Both subjects were sacrificed because of severe symptoms on day 18 post-inoculation. The papulovesicular lesions demonstrated in both monkeys were morphologically similar. Lymph nodes and thymus in both subjects were affected. The most significant differences were that gross lesions with a granulomatous appearance were demonstrated in the gastrointestinal tract organs, such as stomach, small intestine and colon, in the Zr-599-infected monkey, but not in the Liberia-infected monkey (Fig. 1). The peritoneal membrane of the Zr-599-infected subject (#4653) became thickened and had granulomatous lesions, whereas that of the Liberia-infected subject (#4625) was intact. In Zr-599/IN monkeys, one (#4654) showed severe monkeypox-associated symptoms and the other (#4655) showed very mild symptoms. In the Liberia/IN monkeys, the symptoms were relatively mild and of short duration, with only a small number of papulovesicular lesions. The Zr-599-induced ulcerative lesions were still exudative on day 18, whereas the Liberia-induced lesions were dried and covered with scar tissues (Fig. 1). The Zr-599-induced ulcerative lesions seemed to be more severe than the Liberia-induced lesions.

There was a statistically significant difference in virus genome levels as determined by qPCR between Zr-599-infected and Liberia-infected monkeys (see Supplementary Table S2, available in JGV Online), the highest level assessed in combinations of SC and IN group monkeys

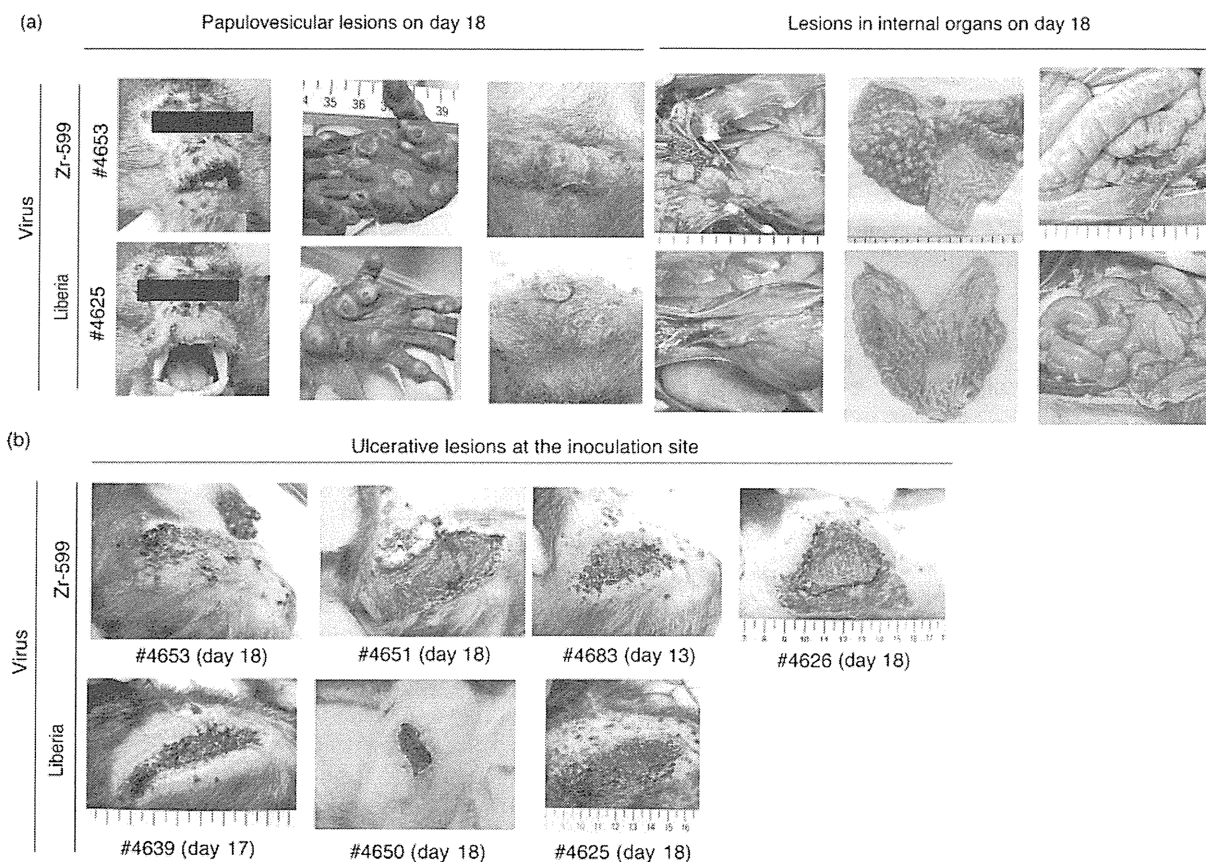

Fig. 1. (a) Monkeypox-associated lesions in skin and internal organs of fatal cases of Zr-599 (#4653) and Liberia (#4625) infection. (b) Ulcerative lesions at the monkeypox virus inoculation site in each subject.

(Student's *t*-test, $P=0.03$) and the mean virus genome level per day in the first 14 days in IN group monkeys (Student's *t*-test, $P=0.02$). All indices in Zr-599-infected monkeys were approximately 10 times higher than those in Liberia-infected monkeys.

There was a significant difference in the IgG response between the Zr-599-infected and Liberia-infected monkeys (Supplementary Fig. S1). In five of the six Zr-599-infected monkeys, an obvious IgG response was demonstrated even on day 10 post-inoculation, whereas the IgG ELISA was negative in all Liberia-infected monkeys except one on day 10. The IgG response was detected 2–3 days earlier in the Zr-599-infected monkeys than in the Liberia-infected monkeys.

The data on monkeypox virus antigen distribution indicated that Zr-599 infected the organs of the respiratory tract, gastrointestinal tract, lymphoid and reticuloendothelial systems, genito-urinary tract and skin, but not the CNS, and that Liberia mainly infected the lymphoid and reticuloendothelial systems and skin (Table 1). Micrographs of immunoperoxidase-stained tissue sections of Zr-599/SC monkeys are available in Supplementary Fig. S2 (in JGV Online).

To compare the severity of monkeypox-associated symptoms, a scoring system was developed and used in this study. The score is composed of two categories: items associated with clinical symptoms and those associated with laboratory findings. The following variables were recorded: decrease in body mass, duration of decreased activity with ill appearance, duration of decreased food consumption (<75 %), body temperature (fever and lower temperature), diarrhoea with/without blood contamination, number of papulovesicular lesions except for the lesions associated with the challenge virus inoculation, outcome, virus genome level (maximum value during the course of observation and the final value when the observation finished), total peripheral blood-cell counts (increased and/or decreased numbers of white blood cells, decreased number of platelets, haemoglobin level) and CRP level. The points for each item are shown in Table 2. It is indicated that the higher the score, the more severe the monkeypox. The mean severity point of monkeypox caused by Zr-599, 25.2, was higher than that caused by Liberia, 17.0. When the same analysis was conducted based on points of the subjects that were infected with virus through the SC route, the mean point of the Zr-599/SC group, 30.0, was higher than that of the Liberia/SC group,

Table 1. Number of subjects with gross monkeypox-associated lesions confirmed by positive monkeypox virus antigen in each organ, as determined by immunohistochemical analyses

Organ	No. subjects with monkeypox virus antigen in each organ			
	Zr-599/SC	Zr-599/IN	Liberia/SC	Liberia/IN
Total *n*	4	2	3	2
Respiratory				
Trachea	3	0	0	1
Lung	3	0	0	2
Cardiovascular				
Heart	0	0	0	0
Gastrointestinal tract				
Liver	4	1	1	0
Pancreas	3	1	0	1
Oesophagus	0	0	0	0
Stomach	3	0	0	0
Ileum	2	1	0	0
Colon	1	0	0	0
Rectum	4	1	0	0
Endocrine system				
Thyroid	2	0	0	0
Adrenal gland	1	0	0	0
Lymphoreticular system				
Radial lymph node	4	1	1	1
Submandibular lymph node	4	2	1	2
Inguinal lymph node	4	1	2	1
Axillar lymph node	4	1	2	2
Tonsil	4	1	2	2
Thymus	4	1	2	2
Spleen	3	1	0	2
Pharyngeal	4	1	1	1
Genito-urinary tract				
Kidney	0	0	0	0
Bladder	1	0	0	0
Prostate/uterus	2	0	1	1
Testis/ovary	3	1	0	0
Skin				
Skin lesions	4	2	3	2
CNS				
Basal ganglia, lateral lobe, frontal lobe, thalamus or spine	0	0	0	0

20.7 (Table 2). The mean point of the Zr-599/IN group, 15.5, was also higher than that of the Liberia/IN group, 11.5.

The pathology of experimental monkeypox virus infections in non-human primates infected with isolate V79-I-005, which was originally obtained from a fatally infected human from Zaire in 1979, through the respiratory route was reported previously (Zaucha *et al.*, 2001). However, the pathology in monkeys infected with monkeypox virus West African strain has not been studied. Monkeypox-associated gross lesions were demonstrated in the following organs: respiratory system, skin, oral cavity, gastrointestinal tract and lymphoid systems. The pathological findings observed in monkeys infected with the Zr-599 strain were similar to those observed in the previous study (Zaucha *et al.*, 2001). The value of the present study is that the pathology of both Congo Basin and West African monkeypox viruses has been investigated. Differences were observed in fatality rate, severity of monkeypox-associated symptoms, virus genome level and the organs affected. Zr-599 replicated in skin, lymphoid and reticuloendothelial systems, genito-urinary tract organs, respiratory tract organs and gastrointestinal tract organs, whereas Liberia replicated only in the skin, lymphoid and reticuloendothelial systems of the monkeys (Table 1). Whilst the lung of a Zr-599/SC-infected monkey was entirely and diffusely affected by the infection (Supplementary Fig. S2), the lung of a Liberia/SC-infected monkey was histopathologically intact (data not shown). Haemorrhagic diarrhoea was observed only in the Zr-599-infected monkeys. These results suggest that the respiratory and gastrointestinal functions were more severely impaired

Table 2. Difference in virulence between the Zr-599 and Liberia strains of monkeypox virus in non-human primates, as determined by the proposed scoring system for analysis of monkeypox severity

Abbreviations: UDL, under detection level; WBC, white blood cells.

Item	No. animals with score	Mean score in each group			
		Zr-599/SC	Zr-599/IN	Liberia/SC	Liberia/IN
Decrease in body mass (%)	<3, 0; 3–<8, 1; 8–<13, 2; >13, 3	2.3±0.5	1.5±0.7	2.3±1.2	2.0±0.0
Recovery signs in body mass	Positive, 0; negative, 3	2.3±1.5	0	1.0±1.7	0
Duration of decreased activity (days)	None, 0; 1–5, 1; 6–10, 2; >10, 3	2.3±1.0	1.5±0.7	2.0±0.0	1.5±0.7
Duration of decreased meal consumption (days)	None, 0; 1–5, 1; 6–10, 2; >10, 3	2.5±0.6	2.0±1.4	2.3±0.6	1.0±0.0
Fever >1 °C	Negative, 0; positive, 1	0.3±0.5	0	0.3±0.6	0
Drop in body temperature >1.5 °C	Negative, 0; positive, 3	2.3±0.5	0	1.0±1.7	0
Faecal appearance	Normal, 0; watery diarrhoea, 1; haemorrhagic diarrhoea, 3	1.8±1.5	1.5±2.1	0.7±0.6	1.0±0.0
Papulovesicular lesions (no.)	None, 0; 1–50, 1; 51–499, 2; >500, 3	2.3±0.5	1.5±0.7	2.0±1.0	1.0±0.0
Outcome	Non-fatal, 0; fatal, 6	4.5±3.0	0	2.0±3.5	0
Maximum virus genome level [$\log_{10}$(copies ml^{-1})]	UDL, 0; <5, 1; 5–7, 2; >7, 3	2.8±0.5	2.5±0.7	2.3±0.6	1.5±0.7
Virus genome level when sacrificed [$\log_{10}$(copies ml^{-1})]	UDL, 0; <5, 1; 5–7, 2; >7, 3	2.0±1.4	1.0±1.4	1.0±1.0	0.5±0.7
Peripheral WBC count <5000 μl^{-1}	Positive, 0; negative, 1	0.5±0.6	1.0±0.0	1.0±1.0	0.5±0.7
Thrombocytopenia <20 000 μl^{-1}	Positive, 0; negative, 1	0.8±0.5	0.5±0.7	0.3±0.5	1.0±0.0
Anaemia with decrease in haemoglobin level >1.5 g dl^{-1}]	Positive, 0; negative, 1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Maximum CRP level [mg dl^{-1}]	<1, 0; 1–<5, 1; 5–<10, 2; >10, 3	2.8±0.5	1.5±0.7	2.3±0.6	1.0±1.4
Mean		30.0±10.4	15.5±9.2	20.7±9.1	11.5±3.5

in the Zr-599-infected monkeys than in the Liberia-infected monkeys.

The difference in the level and course of virus genome detection was consistent with the difference in the pathological findings. Zr-599 replicated more efficiently in the internal organs than did Liberia (Fig. 1; Table 1). The higher level of virus genome detected in the later phase of infection in the Zr-599/SC monkeys might be due to the more efficient replication of Zr-599, particularly in the generalized lymphoid and reticuloendothelial systems, skin, genito-urinary tract organs, respiratory organs and gastrointestinal organs, than that of Liberia in these organs. This feature of Zr-599 might lead to multi-organ failure with malfunctions of respiratory, gastrointestinal and genito-urinary tract organs, resulting in stronger virulence of Zr-599 than of Liberia in non-human primates.

It is believed that Congo Basin strains are more virulent than West African strains in humans, based on clinical studies (Breman *et al.*, 1980; Foster *et al.*, 1972; Ladnyj *et al.*, 1972). Recently, it was reported that a Congo Basin strain, Zr79, which was isolated from a fatal case of monkeypox in Zaire in 1979, was more virulent than the West African strain US03, which was isolated from a non-fatal case of monkeypox in the USA in 2003, using a ground squirrel model for monkeypox virus infection (Sbrana *et al.*, 2007). The clinical course and virological and pathophysiological features of monkeypox virus infections in non-human primates obtained in this study were different from those reported in the ground squirrel model (Sbrana *et al.*, 2007; Tesh *et al.*, 2004). Monkeypox virus infections in non-human primates resemble human monkeypox in terms of pathophysiological profile, making the present study of particular value.

The genetic and molecular mechanism(s) underlying the differences in pathogenesis between Congo Basin and West African strains should be clarified through further studies. It was reported that *D10L*, *D14L*, *B10R*, *B14R* and *B19R* were possibly responsible genes, with *D14L*, an orthologue of vaccina complement protein, as a leading candidate and with *D10L* and *B19R* as less likely candidates (Chen *et al.*, 2005). Although the data are not shown here, the *D14L* gene was confirmed to be absent in the Liberia strain, as in the case of an West African strain, SL-70 (Chen *et al.*, 2005).

In conclusion, it was demonstrated that Zr-599, a Congo Basin strain, was more virulent than Liberia, a West African strain. The difference in virulence might be due to the difference in the sites of virus replication resulting in organ dysfunction: Zr-599 replicated in skin, lymphoid and reticuloendothelial systems, genito-urinary tract organs, respiratory organs and gastrointestinal organs, whereas Liberia replicated only in skin, lymphoid and reticulo-endothelial systems.

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Characterization of Monoclonal Antibodies to Junin Virus Nucleocapsid Protein and Application to the Diagnosis of Hemorrhagic Fever Caused by South American Arenaviruses▽

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Junin virus (JUNV), Machupo virus, Guanarito virus, Sabia virus, and Chapare virus are members of New World arenavirus clade B and are the etiological agents of viral hemorrhagic fevers that occur in South America. In this study, we produced three monoclonal antibodies (MAbs) to the recombinant nucleocapsid protein of JUNV, designated C6-9, C11-12, and E4-2. The specificity of these MAbs was examined by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay, and an epitope-mapping method. Using these MAbs, we developed antigen (Ag) capture ELISA systems. We showed that by using MAb C6-9, JUNV Ag was specifically detected. On the other hand, by using MAb C11-12 or E-4-2, the Ags of all human pathogenic South American arenaviruses were detected. The combined use of these Ag capture ELISA systems in the present study may be useful for the diagnosis of acute-phase viral hemorrhagic fever due to infection by a South American arenavirus.

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The South American arenaviruses Junin virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Sabia virus (SABV), and Chapare virus (CHPV) are members of New World arenavirus clade B. JUNV, MACV, GTOV, and SABV are the etiological agents of Argentine hemorrhagic fever (AHF), Bolivian hemorrhagic fever (BHF), Venezuelan hemorrhagic fever (VHF), and Brazilian hemorrhagic fever, respectively (4). CHPV was also recently shown to be associated with cases of hemorrhagic fever in Bolivia (5). AHF emerged in the 1950s, and since then, outbreaks have occurred annually without interruption (4). The mortality rate for AHF is estimated to be 15 to 30%, but early treatment with immune plasma reduces the rate to less than 1% (6). The region at risk has been progressively expanding into northern central Argentina, and almost 5 million people are currently considered to be at risk for AHF (6, 13). Phylogenetic analysis indicates that JUNV is more closely related to MACV than to SABV or CHPV, whereas SABV and CHPV are more closely related to each other than to other New World arenaviruses (5).

Arenaviruses are enveloped and contain a bisegmented RNA genome. The genome consists of two ambisense single-stranded RNA molecules, one designated L, which encodes the RNA-dependent RNA polymerase and a zinc-binding matrix protein, Z, and the other designated S, which encodes the major structural components of the virion, i.e., the nucleocapsid protein (NP) and the envelope glycoprotein precursor (15). The arenavirus NP is the most abundant protein among the viral structural proteins both in infected cells and in virions (2) and is commonly used as a target for detecting viral antigens (Ags) (20). Moreover, arenavirus NPs have been known to be the most conserved among the same virus species and, to some extent, among different arenavirus species (3, 8). Therefore, it seems likely that monoclonal antibodies (MAbs) raised against the NP of an arenavirus would also be useful for detecting other arenaviruses (20). Recently, an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was developed by using a recombinant NP (rNP) of JUNV, obtained from a recombinant baculovirus system, and was proposed to be useful for etiologic confirmation of AHF in seroepidemiological studies (20, 26). It is considered that an Ag capture ELISA using MAbs specific for viral Ags allows rapid diagnosis of the acute phase of viral hemorrhagic fever by detecting viral Ags in blood or tissue homogenates (20). In this study, we produced MAbs to the rNP of JUNV. These MAbs were characterized by ELISA, indirect immunofluorescence assay (IFA), and an epitope-mapping method. Ag capture ELISAs were developed by using these MAbs that are specific for JUNV and that are broadly applicable for the detection of human pathogenic New World arenaviruses.

MATERIALS AND METHODS

Cell culture. Hybridomas and their parental cell line, P3/Ag568, were maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (Invitrogen), and antibiotics (streptomycin and penicillin G; Invitrogen). Hypoxanthine-aminopterin-thymidine supplement (Invitrogen) was added to the me-

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dium for selection of hybridomas, as recommended by the supplier. BTI-TN-5B1-4 (High Five; Invitrogen) insect cells were maintained in TC100 (Invitrogen) supplemented with 10% FBS, 2% tryptose phosphate broth (Difco, Detroit, MI), and kanamycin (Invitrogen). HeLa cells were maintained in minimal essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FBS and antibiotics (streptomycin and penicillin G; Invitrogen).

Recombinant baculoviruses. The baculoviruses Ac-JUNV-NP and Ac-His-Lassa virus (LASV)-NP, expressing the JUNV and His-LASV rNPs, respectively, were generated as described previously (20).

The cDNAs of the MACV, GTOV, SABV, and CHPV NPs were obtained by chemical synthesis (Codon Devices, Cambridge, MA). The GenBank accession numbers of the nucleotide sequences of the MACV, GTOV, SABV, and CHPV NP genes are NC_005078, AF485258, NC_006317, and NC_010562, respectively. The cDNAs of the MACV, GTOV, SABV, and CHPV NPs were digested with BamHI and subcloned into the BamHI restriction site of pAcYM1 (14), and the resulting plasmids were designated pAcYM1-MACV-NP, pAcYM1-GTOV-NP, pAcYM1-SABV-NP, and pAcYM1-CHPV-NP, respectively. High Five cells were transfected with mixtures of linearized BacPAK6 DNA (Clontech, Mountain View, CA) and the recombinant transfer vector according to the manufacturer's instructions and the procedures described by Kitts and Possee (10), and recombinant baculoviruses were obtained from them. The baculoviruses expressing the MACV, GTOV, SABV, and CHPV rNPs were designated Ac-MACV-NP, Ac-GTOV-NP, Ac-SABV-NP, and Ac-CHPV-NP, respectively.

Expression and purification of rNPs. High Five cells infected with Ac-JUNV-NP, Ac-MACV-NP, Ac-GTOV-NP, Ac-SABV-NP, Ac-CHPV-NP, or Ac-His-LASV-NP were incubated at 26°C for 72 h. The cells were then washed twice with cold phosphate-buffered saline (PBS) solution. The High Five cells were lysed in PBS containing 1% NP-40 and 2 M urea. After the cell lysates were centrifuged at 15,000 × *g* for 10 min, the pellet fractions were collected and then solubilized in PBS containing 8 M urea. After the samples were centrifuged, the supernatant fractions were used as the purified Ags. The control Ag was produced from High Five cells infected with Ac-ΔP, which lacks the polyhedrin gene, in the same manner as for the negative control Ags. All Ags were aliquoted and kept at −80°C until use.

Establishment of MAbs. BALB/c mice were immunized three times with the purified JUNV rNP. Spleen cells were obtained 3 days after the last immunization and fused with P3/Ag568 cells by using polyethylene glycol (Invitrogen). The culture supernatants of the hybridoma cells were screened by ELISA with purified JUNV rNP as an Ag in the presence of 2 M urea. MAbs were purified from the culture supernatant by using a MAbTrap GII antibody purification kit (GE Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. The concentration of each purified MAb was also determined by use of a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Polyclonal antibodies. Polyclonal antibodies were induced in rabbits by immunization with the purified rNPs of JUNV, MACV, GTOV, SABV, and CHPV, respectively. Rabbit sera collected before immunization were used as controls.

IgG ELISA. The IgG ELISA was performed as previously described, except for Ag preparation (20–22). Briefly, ELISA plates (96 wells, Pro-Bind; Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with the predetermined optimal quantity of purified JUNV, MACV, GTOV, SABV, CHPV, or His-LASV rNP (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was covered with 200 μl of PBS containing 5% skim milk and 0.05% Tween 20 (PBST-M), followed by incubation for 1 h for blocking at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and then inoculated with MAbs (100 μl/well), which were diluted 1:1,000 with PBST-M. After a 1-h incubation period, the plates were washed three times with PBST and then the plates were inoculated with goat anti-mouse IgG antibody labeled with horseradish peroxidase (HRP; 1:1,000 dilution; Zymed Laboratories, Inc., South San Francisco, CA). After a further 1-h incubation period, the plates were washed and 100 μl of ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at room temperature, and the optical density at 405 nm (OD_{405}) was measured against a reference of 490 nm. The adjusted OD_{405} value was calculated by subtracting the OD_{405} value of the negative Ag-coated wells from that of the corresponding wells.

IFA. The full-length cDNA of the JUNV NP obtained from Ac-JUNV-NP, which possessed a BamHI restriction site at both extremities, was cloned into the BamHI site of the pKS336 vector (23), and the resulting plasmid was designated pKS-JUNV-NP. Also, the chemically synthesized full-length cDNAs of the MACV, GTOV, SABV, and CHPV NPs were cloned into the BamHI site of the pKS336 vector and the resulting plasmids were designated pKS-MACV-NP, pKS-GTOV-NP, pKS-SABV-NP, and pKS-CHPV-NP, respectively. HeLa cells were then transfected with each of these expression plasmids by using a transfection reagent (FuGENE6; Roche Diagnostics) according to the manufacturer's instructions. The transfected cells were selected with 4 μg of blasticidin *S*-hydrochloride/ml in culture medium. The HeLa cell clones were obtained by analyzing the expression of each rNP by IFA with rabbit serum raised against the JUNV, MACV, GTOV, SABV, or CHPV rNP, as previously described (20).

Ag capture ELISA. Purified MAb C6-9, C11-12, or E4-2 was used to coat microwell immunoplates (Falcon; Becton Dickinson Labware) at 100 ng/well in 100 μl of PBS at 4°C overnight, followed by blocking with PBST-M for 1 h at room temperature. After the plates were washed with PBST, 100 μl of samples containing serially diluted rNP of JUNV, MACV, GTOV, SABV, CHPV, or LASV was added and the plates were incubated for 1 h at 37°C. The plates were then washed with PBST, and 100 μl of rabbit polyclonal antibody raised against the rNP of JUNV diluted 1:500 with PBST-M was added to each well. After 1 h of incubation at 37°C, the plates were washed with PBST, and HRP-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) was added. The plates were incubated for 1 h at room temperature. After another extensive washing with PBST, 100 μl of ABTS substrate solution (Roche Diagnostics) was added and the OD_{405} was measured with a reference wavelength of 490 nm after 30 min of incubation at room temperature. As a negative control, the OD of control Ag-inoculated wells was measured. The adjusted OD_{405} values were calculated by subtracting the OD_{405} value of the negative control well from the corresponding OD_{405} values. Means and standard deviations were calculated from the ODs of 12 negative control wells, and the cutoff value for the assay was defined as the mean plus 3 standard deviations.

Expression of truncated rNPs of JUNV. In order to determine the epitope on the JUNV rNP for the MAbs, a series of truncated JUNV rNPs were expressed as fusion proteins with glutathione *S*-transferase (GST). The DNA corresponding to each of the truncated NP fragments was amplified by PCR with specifically designed primer sets. The amplified DNA was subcloned into the BamHI and EcoRI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, England). The GST-tagged full-length rNP (GST-JUNV frNP) or truncated forms of the rNP (GST-JUNV trNPs) were expressed in *Escherichia coli* BL21 and then partially purified.

Western blotting. The MAbs were tested for reactivity to GST-JUNV frNP and a series of GST-JUNV trNPs by Western blotting as reported previously (9, 18, 24).

MAb epitope mapping. The epitopes for MAbs C6-9 and C11-12 were determined by epitope-blocking ELISA using synthetic peptides. The decapeptides were chemically synthesized by shifting one amino acid, with a consecutive overlap of nine amino acids to cover the JUNV NP (amino acids [aa] 5 to 26 for C6-9 and aa 543 to 564 for C11-12). ELISA plates were coated with purified JUNV rNP prepared by using a baculovirus expression system (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was inoculated with 200 μl of PBS-M, followed by incubation for 1 h for blocking. MAb C6-9 or C-11-12 was mixed with each peptide (1 μg/well) and incubated for 1 h at 37°C, and then the mixture was added to each well of the plates. After a 1-h incubation period, the plates were washed three times with PBST, and then the plates were inoculated with goat anti-mouse IgG antibody labeled with HRP (1:1,000 dilution; Zymed). The following procedure was performed as described in the IgG ELISA section above.

For MAb E4-2, the epitope was determined by ELISA using GST-JUNV frNP and trNPs. ELISA plates were coated with purified GST-JUNV frNP or trNPs (approximately 100 ng/well) according to the method described in the IgG ELISA section above. MAb E4-2 or an anti-GST MAb was used for detection at a 1:2,000 or a 1:500 dilution, respectively. The adjusted OD_{405} was calculated by dividing the OD_{405} of MAb E4-2 by that of the anti-GST MAb from the corresponding wells.

RESULTS

Generation of MAbs. In order to obtain MAbs against the JUNV NP, BALB/c mice were immunized with the purified rNP of JUNV. The MAbs were purified and tested for reactivity to the rNP of JUNV by IgG ELISA. Three MAbs, designated MAb C6-9, MAb C11-12, and MAb E4-2, reacted with the rNP of JUNV by IgG ELISA even in the presence of 2 M urea.

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