的検索として、細胞から抽出したmRNAを用いてReal-timePCRを行った。生化学的検索として、上清を用いてELISAを行い、細胞を用いてWesternblotによる検索を行った。
検索対象は、AGが産生・放出する神経栄養因子であるNGF、BDNFおよびGDNFと、そのレセプターである。
2）実験1で得られた結果（後述）を確認するため、神経細胞に分化させた株化ヒト神経芽細胞（SH-SY5Y）を用いて以下の実験を行った。まず培養液中にAβ1-42を10μMの濃度にて添加し、3時間後に各神経栄養因子を培養液中に加える。その後、Aβ添加より24時間後におけるシナプスの状態を生化学的ならびに免疫組織化学的に検索を行った。

C. 結果

1）Real-timePCRの結果より、Aβ添加後3時間において、AGにおけるBDNFの遺伝子発現が有意に上昇していることが明らかになった。NGF、GDNFはほぼ変化が見られず、24時間後においても両者の遺伝子発現様式に変化は無かった。蛋白レベルでのBDNF発現・産生上昇を確認するため、サンドイッチELISA法を用いて検索を行ったところ、蛋白レベルにおいてもAβによってBDNFの産生が有意に上昇していることが明らかとなった。尚、24時間後においては、このBDNF発現・産生上昇は収束していた。次いで、BDNFのレセプターに関する検索を行ったところ、大脳皮質初代培養系（90%以上がニューロン）ではAβ添加3時間後にTrkBの発現上昇が確認された。しかしながら、AGのTrkB発現レベルはほぼ一定であったことから、上記BDNF産生上昇はニューロンに対するものであることが明らかになった。BDNFはTrkB以外にもp75NTRというレセプターを持ち、前者が神経細胞保護的な効果を持つのに対し、後者はアポトーシス誘発性に働く。そこでp75NTRについても同様の検索を行ったところ、Aβ添加3時間後において、p75NTRの発現が有意に低下していることが明らかになった。この結果から、上記BDNF産生上昇はニューロンにアポトーシスを促す物ではなく、保護的に働いている物であることが明らかとなった。
2）実験1で確認されたBDNF産生上昇が実際にAβによるニューロンの障害を緩和するか否かを確認するために、ヒト由来培養系を用いた評価実験を行った。その結果、BDNF添加群においては、SynapsinIおよびSynaptophysinの蛋白レベルが、Aβのみ添加した群と比較して有意に保存されており、免疫染色の結果からも、シナプス構築が維持されていることが明らかになった。

D. 考察

本研究によって、Aβ毒性に対してAGが特定の神経栄養因子を放出することが初めて明らかとなった。過去の臨床報告において、AD患者脳ではBDNFの発現が低下していることが明らかとなっているが、本研究において我々は、BDNFがAβに

よるシナプス障害・変性からニューロンを保護することを証明した。このことから、Aβ誘発性シナプス障害とBDNFによる（＝AGによる）保護機能の間には密接な関係が存在する可能性が考えられる。このAβによるシナプス障害は、AD予備群と目されている軽度認知障害の原因ではないかと考えられており、これら障害の更なる重篤化がアルツハイマー病を主とする認知症発症の引き金になるのではないかと示唆されている。従って、本研究によって明らかとなったAGのAβ反応性BDNF産生上昇機能を上手く利用できれば、薬物治療ではなく患者自身の治癒力を用いた新たな予防法・治療法の開発が可能になると考えられる。
ただし、Aβ添加24時間後（この時間帯においては、シナプスは崩壊している）では、AGにおけるBDNFの発現・産生上昇やニューロンにおけるTrkB発現上昇は確認されなかったことから、今回明らかになったAGの機能は、ニューロンやAGがAβ毒性によって完全に破壊・変性していないことが条件であるとも考えられる。

E. 結論

本研究においてAGの新たな対Aβ反応性神経細胞保護機能が明らかとなったことから、今後AGがAD病態制御の新たなターゲットとして働く可能性が示唆された。

最近のAD治療法開発においては、主にAβ切断酵素の阻害薬やAβ免疫療法が注目を浴びているが、それらが実際にニューロンを保護するか否かは未だ不明である。また、全AD患者の80%以上は加齢性に発症する孤発性であるが、孤発性患者の脳内ではAβ産生量は健常老人とほぼ変わらないことも知られている。本研究によって我々は、BDNFが、少なくともin vitroにおいてはAβ毒性によるシナプス障害からニューロンを保護することを明らかにしたことから、新たな治療法の一つになるのではないかと注目している。そこで今後は、in vivoにおけるBDNFの対Aβニューロン保護作用を確認する実験を行う予定である。

F. 健康危険情報

特になし。

G. 研究発表

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H. 知的財産権の出願・登録状況
特になし。

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分担研究報告書

画像データベースを用いた基盤技術開発
～カニクイザルにおける循環器疾患モデル研究への応用～

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研究要旨

昨今の医科学研究においてはサル類を用いた有効性・安全性評価の需要が今後ますます増え続ける事が予想され、画像診断を用いた評価基準の樹立が必須となる。将来予想されるサル類を用いた前臨床試験の有効性・安全性評価に画像診断を適応する目的で、サル類における MRI、超音波検査、X 線検査などによる画像診断技術を確立し、画像データベースの構築を進め、サル類の研究基盤技術整備を行った。特に実験用サル類においては心疾患の系統だった検査はこれまでほとんど報告されていないことから、サル類における心機能評価基準の樹立も目的としてカニクイザル繁殖コロニー内のスクリーニングにも着手し、心室中隔欠損症、右室二腔症、弁膜症などの心疾患モデルの抽出にも一部成功した。

キーワード：画像診断、超音波検査、MRI、循環器疾患、心室中隔欠損、右室二腔症、弁膜症

A．研究目的

再生医療・遺伝子治療・創薬研究などにおいてはサル類を用いた有効性・安全性評価の需要が今後ますます増え続ける事が予想される。それら評価の方法としては画像診断を用いた評価系の樹立が必須となる。将来予想されるサル類を用いた前臨床試験の有効性・安全性評価に画像診断を適応する目的で、サル類における MRI、超音波診断等を用いた画像診断技術を確立し、画像データベースの構築、評価基準の樹立を進め、サル類の研究基盤技術整備を行うことを目的とする。さらにこれらの技術、評価基準を確立することにより、繁殖コロニー内の先天性の疾患モデルザルの抽出が可能となる。

特に実験用サル類においては系統だった心機能検査、循環器疾患の生前診断例はこれまでほとんど報告されていないことから、サル類における心機能評価基準の樹立も目的としてカニクイザル繁殖コロニー内の循環器疾患スクリーニングも試みる。人と遺伝的に近縁なサル類での心機能解析、心疾患モデルの抽出、病態解析は心臓病態学的にも極めて重要なものとなる。

B．研究方法

カニクイザル繁殖コロニーおよび

Aging farm を対象にして塩酸ケタミン麻酔下で超音波、心電図、X線、MRIなどの非侵襲的な各種検査を行い、画像診断に必要な技術を樹立する。これらの諸検査のうち特に超音波検査は心臓内腔の観察が可能であるため異常個体の確定診断を行うことが可能である。また循環器疾患時の内分泌調節機構である心臓血管ホルモン(心房性ナトリウム利尿ペプチドおよび脳性ナトリウム利尿ペプチド)はヒトにおいても重要な臨床的指標となっていることからサルでの指標化も目指し、測定を実施する。これら得られた検査結果より画像を中心にしたデータベースを構築することで評価基準、特に心機能の評価基準を樹立する。その上で評価基準に照らし総合的な画像診断を行う事により自然発生疾患の抽出を目指す。疾患の疑われる死亡症例や検査で得られた症例の症状によっては安楽殺を検討し、病理組織学的検索も試みる。

C. 研究結果

まず、3TMRIを用いてT1,T2,Pefusion, Diffusion 画像の撮像を目的としたカニクイザル脳用シークエンスの開発を行った。循環器疾患を対象に MRI を用いた心電図同期撮像法および造影法を確立し、心機能評価、心筋血流評価など心疾患モデルの評価系樹立の基礎を構築した。さらに超音波、X線、心電図の検査技術を樹立し、繁殖コロニーを対象とした自然発生疾患のスクリーニングにも一部着手し、画像データベースの構築、評価基準の樹立を行った。

スクリーニングの結果、評価基準に照らし心室中隔欠損症、右室二腔症、弁膜症などの心疾患を抽出することに成功した。下記にそれら疾患の詳細を記す。

心室中隔欠損では心エコー図において心室中隔筋性部より右心室内へ流入する乱流像が確認され、ヒトにおいてもまれである筋性部心室中隔欠損であることが確定診断出来た。その他にも膜性部に欠損を認める同疾患も抽出した。

右室二腔症では胸部エックス線検査において右心系の拡大所見、心エコー図検査では右室流出路に高エコーの腫瘤塊が認められた。死亡後の心臓肉眼所見では肺動脈弁下に腫瘤塊を確認し、組織学的検査で腫瘤塊は血栓であることが確認された。血漿心房性ナトリウム利尿ペプチドは 49.9 pg/ml から状態の悪化により 106 pg/ml へ上昇し、血漿脳性ナトリウム利尿ペプチドも 19.1 pg/ml から 67.3 pg/ml へ上昇した。

25 歳という高齢ザルでは僧帽弁に 3.76m/sec の逆流波を連続波ドプラ法により検出し、加齢性変化と思われる弁膜障害による軽度な僧帽弁逆流症が抽出された。本疾患では心エコー所見、剖検所見においても僧帽弁の肥厚を確認した。

なお、本結果を含む研究流れ図を末尾に添付する。

D. 考察

今回の研究によりサル類における MRI、X線、超音波検査等の画像診断技術、画像データベースを構築し、心機能評価基準を樹立することでカニクイザル基盤高度化の一役を担えたものと思われる。特に、心エコー図検査および心臓血管ホルモン測定はサル類の心機能検査においても有効な診断手法であることが示唆された。また得られた技術、データベースを用いて、サル類においてはほとんど報告のない自然発生循環器疾患の生前診断にも成功し、種々の疾患モデルを抽出することが可能であることを証明した。すなわち、これらは当カニクイザル繁殖コロニー内には心臓病態学的にも重要な循環器疾患モデルが多数存在することを示唆するものであると思われる。

E. 結論

本研究においてサル類の画像診断技術、画像データベース、評価基準を樹立することにより医科学研究に有用な基盤技術の確立を行う事が出来た。また、それらを用い一部循環器疾患モデルの抽出にも成功した。今後は、サルを用いた前臨床試験の有効性・安全性評価を行うのにより十分な画質と解像度を得るべく、引き続きサルに特化した様々な画像診断技術、確定診断法、データベースのさらなる充実を図る事が必要となる。さらに、本研究を継続することで、より多くのカニクイザル疾患モデルの抽出が可能となり、抽出された疾患の詳細な病態解析をなせる事は貴重なリソースの樹立にもつながるものと思われる。特にサル繁殖コロニーの特性を生かし、遺伝的調査ならびに横断的調査を行えることで、サル類の心疾患の現状を明らかにし、心臓病態学のみならずサルを用いた様々な医科学研究への貢献をできる点は本研究ならではである。

F. 健康危険情報

特になし。

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H. 知的所有権の出願・登録状況

なし

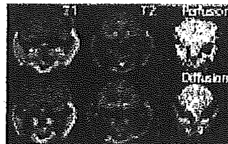
画像データベースを用いた基盤技術開発
～カニクイザルにおける循環器疾患モデル研究への応用～

遺伝子治療・再生医療などの新規治療法開発研究、有効性・安全性評価のためにはサルモデルが必須。

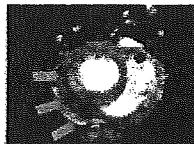
- ❑画像診断による客観な安全性・有効性評価が必須
- ❑サル類における疾患の生前画像診断の報告例はほとんど無い
- ❑サルモデルの評価基準の確立がなされていない

サル類における画像診断技術を確立し、疾患モデルの評価系樹立、抽出

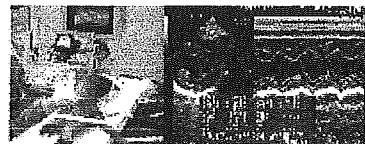
画像診断技術の確立



❑ カニクイザル脳用シークエンスの開発



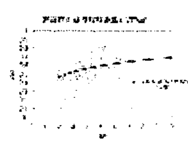
❑ MRIを用いた心電図同期撮像法および造影法を確立

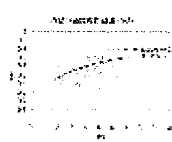


❑ 超音波診断などを用いた心疾患スクリーニング法の確立

データベース・評価基準の樹立

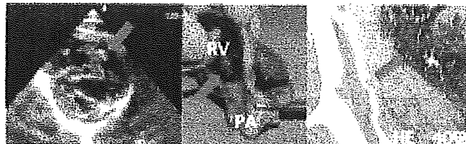
❑ MRI画像のデータベース



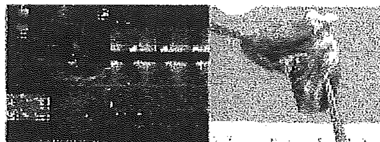


❑ 超音波診断より得られた各個体の心室中隔、左室自由壁壁厚は体重との相関をよく認め、算出された駆出率などを評価基準とした

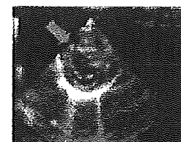
疾患の抽出



❑ 右室二腔症：心エコー図検査で右室流出路に高エコーの腫瘤塊、死亡後の心臓肉眼所見では肺動脈弁下腫瘤塊が形成され、組織学的検査で腫瘤塊は血栓であることが証明された



❑ 僧帽弁逆流症：僧帽弁に逆流波を連続波ドプラ法により検出し、剖検においても僧帽弁の肥厚を確認



❑ 心室中隔欠損症：心エコー図において心室中隔筋性部より右心室内へ流入する乱流像を確認

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Survey of Captive Cynomolgus Macaque Colonies for SRV/D Infection Using Polymerase Chain Reaction Assays

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The exogenous simian type D retroviruses (SRV/Ds) are prevalent in macaque monkeys and sometimes cause immunodeficiency with anemia, weight loss, and persistent unresponsive diarrhea. SRV/D isolates are classified as subtypes 1 to 6, and the entire sequences of the *gag* region of SRV/D-1, -2, and -3 and SRV/D-Tsukuba (SRV/D-T) have been determined. We designed specific primers in the *gag* region of SRV/D-T that enabled us to directly detect by polymerase chain reaction (PCR) SRV/D-T proviral DNA sequences in DNA extracted from whole blood. Using this assay and another PCR assay that detects multiple SRV/D subtypes, we performed a survey for SRV/D infection in our specific pathogen-free (SPF) and conventional colonies at Tsukuba Primate Center (TPC). In the SPF colony, no SRV/D signal was detected in any animal. On the other hand, SRV/D-T was detected in 11 of 49 animals (22.5%) in the conventional colony. SRV/D-T was the only SRV/D subtype detected. Consequently, SRV/D-T is the major SRV/D subtype present in cynomolgus monkeys at TPC.

The exogenous simian type D retroviruses (SRV/Ds) are prevalent in colony-born macaques and sometimes cause immunodeficiency with anemia, weight loss, and persistent unresponsive diarrhea (2, 3, 5, 12). Macaques are needed for a variety of biomedical studies such as preclinical studies in regenerative medicine, safety testing for vectors in gene therapy, and developing AIDS vaccines using simian immunodeficiency virus (SIV or SHIV) (1, 9, 13).

If macaques infected with SRV/D are used in such experiments, the results may be affected by the animal's condition due to the onset of clinical signs of SRV/D induced diseases including immunodeficiency, and in the worst case, increased mortality may result in the loss of research monkeys (9). Therefore, the eradication of SRV/D from macaque breeding colonies is of great importance. Unlike other viral agents (e.g., herpesviruses and measles virus), some monkeys may carry SRV/D without having detectable serum antibodies. Therefore, it is necessary to perform both serologic testing and detection of viral presence. Because virus isolation is time- and labor-intensive, detection of SRV/D proviral DNA using polymerase chain reaction (PCR) technology has been used as a rapid and effective testing method. In addition, PCR tests that simultaneously detect multiple SRV/D serotypes have been reported (8, 23).

SRV/Ds are classified into subtypes 1 to 6, and the entire genome sequences of SRV/D-1, -2, and -3 have been determined (17, 19, 21). Recently, part of the *gag-prt* sequence of SRV-5 was determined (11), and SRV/D-6 has been isolated from a Hanuman langur (*Semnopithecus entellus*) (15). We recently described the isolation of SRV/D-T from cynomolgus monkeys in the breeding colony at the National Institute of Infectious Diseases (NIID; Tokyo, Japan) and reported the sequence of the *gag* region of this isolate (4). The SRV/D-T subtype was isolated from two cynomolgus monkeys in the conventional breeding colony at TPC. However, it is not known whether this subtype was isolated by chance or if it is prevalent in the colony. In order to survey the SRV/D subtypes prevalent in our breeding colony, we designed an SRV/D-T-specific PCR primer set based on the sequences of the *gag* regions of SRV/D -1, -2, and -3 and SRV/D-T. To increase the sensitivity of PCR detection, an SRV/D-T-specific nested primer set also was designed. Here, we report the extent of infection with SRV/D in cynomolgus monkeys from our specific pathogen-free (SPF) and conventional colonies by using a direct PCR detection method with ethlylenediamine tetraacetic acid (EDTA)-treated whole blood and the SRV/D-T-specific primer set and other published primer sets that detect multiple SRV/Ds.

Materials and Methods

Humane care and use of animals. All procedures involving the use of animals in this study conformed to the Rules for Animal Care and Management of Tsukuba Primate Center (6) and the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan (18). Housing and care procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Colony description. For initial establishment of the indoor cynomolgus monkey breeding colony at TPC, monkeys (*Macaca fascicularis*) were imported from the Philippines, Indonesia, and Malaysia from 1979 to 1983. Since this time, most monkeys have

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Table 1. Polymerase chain reaction (PCR) primer sets for detection of simian type D retroviruses (SRV/D)

Name	Sequence (5' to 3')	Product size (bp)	Subtype detected	Region
Tga1[a] Tga2	GGGCTAAAGCCTCGACAA AGACTTCCACAGTAGGTGAT	722	SRV/D-T	gag
Tga3[a] Tga4	GAAGAAAAAGGTACCCAATGA GGCACAGGAGAAGAGTCTTT	381	SRV/D-T	gag
SRVPOLF1[b] SRVPOLR1	TACCAITICCTCATTTIGGAGTTAATCC AAAGAGTGCITGATTGAGIATITTCCTG	446	SRV/D-1 to -5	pol
SRVPOLF2[b] SRVPOLR2	RITICCCAAIAATGITITGGCAAATGGA AGGIIGICCAAATAATAGAGAAGCAATG	181	SRV/D-1 to -5	pol
WH1[c] WH3	CCGCTGTGATGGCGGTAGTCAATCC CTGGGAAAATATCCTTGGGAGGATATTC	268	SRV/D-2	gag
RC14[c] RC15	CAATCCTAAAGAGGAACTCAAAGA TATTCTTCTGTGTTTTTATTAATAAGG	222	SRV/D-2	gag
WH1[c] WH3	CCACTGTAATGGCGGTTGTTAATCC CTGGGAAAATATCTTTTGGGATTTGTTC	268	SRV/D-1 and -3	gag
RC12[c] RC13	TAATCCAAAAGAGGAGCTCAAAGA TTGTTCTTCCTTTTCTCGACTGGC	222	SRV/D-1 and -3	gag

[a]SRV/D-T-specific primer set.
[b]The generic primer set for amplifying SRV/D-1 through -5 (10).
[c]The primer sets for simultaneous detection of SRV/D-1,-3, and -2 (8).

been kept in individual cages set in high-efficiency particulate air (HEPA)-filtered, negative-pressure chambers maintained at 23 to 27°C and 50 to 70% humidity with 12 air changes/h. For breeding, a timed mating system is used in which female monkeys are placed in the male macaque's cage for at least 3 days, and pregnancy is confirmed by ultrasonography. About 4 months after birth, infants are separated from their mothers and paired with an infant of similar size. This colony supplies approximately 180 monkeys, including juvenile monkeys and retired breeders, for research every year.

The conventional cynomolgus monkey group used in these studies consisted of 49 retired breeders (21 male and 28 female) ranging in age from 15 to 37 years; the average numbers of mating partners of these monkeys were 25.4 female macaques per male and 7 male monkeys per female, with each female having 0 to 10 (average, 2.8) offspring. These retired breeders were chosen as representatives of our conventional breeding colony in this study for two reasons. First, given the close contact of these animals with many others over several years, if a virus is present in the colony, these individuals should have acquired it. Second, the group size was similar to that of the SPF colony.

Microbiological status. Periodic health examinations of all monkeys in the breeding colony includes serum biochemistry and microbiological testing every 2 years. Bacteriological testing for *Shigella*, *Salmonella*, and mycobacteria was performed on arrival from Southeast Asia. Simian varicella virus (SVV) has been eradicated from the conventional breeding colony on the basis of antibody testing (14). Serological screening for B virus uses an enzyme immunoassay from BioReliance (Rockville, Md.), with follow-up surveillance conducted every 2 years using both SA8 and B virus antigens (20). Therefore, monkeys in the conventional breeding colony are free from *Shigella*, *Salmonella*, mycobacteria, B virus, and SVV.

Besides the conventional cynomolgus monkey breeding colony, TPC maintains a small SPF colony that is free of SRV/D in addition to B virus and SVV. For SRV/D screening, antibody testing is done by Western blotting with SRV/D-2 antigen (BioReliance), and virus isolation uses the Raji cell syncytial assay (2). SRV/D-negative monkeys were transferred into an SPF room (HEPA-filtered and positive pressure). Follow-up surveillance by antibody testing and virus isolation was conducted every 4 months for the first year and every 6 months thereafter.

Template DNAs. Genomic DNA from macaque whole blood treated with EDTA was prepared using a NucleoSpin Blood kit (Macherey-Nagel, Duren, Germany). For testing the specificity of primers, genomic DNA from Raji cells infected with SRV/D-T (Tsukuba isolate) was extracted using the NucleoSpin Blood kit. Genomic DNAs from Raji cells infected with SRV/D-1 and SRV/D-2 were kindly supplied by Dr. N.W. Lerche (California National Primate Research Center, Davis, Calif.). Plasmid DNA containing the SRV/D-T *gag* region fragment (2189 bp) (4) was prepared using a QIAprep Spin miniprep Kit (Qiagen, Tokyo, Japan).

PCR. Primers specific for SRV/D-T were designed based on the aligned SRV/D-1, -2, and -3 and SRV/D-T sequences (GenBank accession nos. M11841, AF126467, M12349, and AB181392, respectively; Table 1). Primers used for simultaneous detection of multiple SRV/D subtypes (8, 10) also are listed in Table 1. First-round PCR amplification was performed with 500 ng of genomic DNA, 20 pmol of each primer, 10 mM, Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 units Takara Ex *Taq* polymerase (TaKaRa, Otzu, Japan) in a total volume of 50 μl. The amplification was performed as follows: initial denaturation at 95°C for 5 min; 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 10 min. These conditions were used for primer evaluation and for mixed PCR testing. Amplified PCR products were visualized by electrophoresis in 1.2% agarose gels stained with 0.5 mg/ml ethidium bromide under ultraviolet transillumination.

Nested PCR was performed with 2 μl of the first round PCR reaction mixture, 20 pmol of each primer, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 units of Ex *Taq* polymerase (TaKaRa) in a total volume of 50 μl. The am-

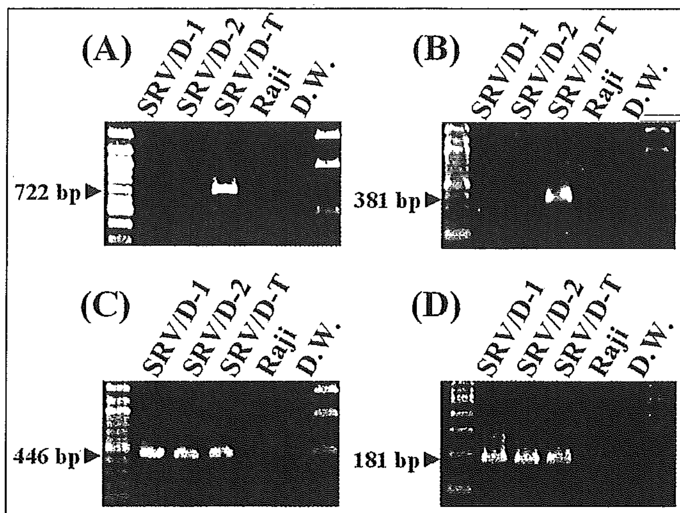

Figure 1. Specific detection of simian type D retrovirus (SRV/D)-T proviral DNA in genomic DNA from Raji cells infected with SRV/D-1, -2, or -T by nested polymerase chain reaction (PCR) assays. (A) Agarose gel electrophoresis of first-round PCR products with the SRV/D-T-specific primers Tga1 and Tga2. (B) Agarose gel electrophoresis of nested PCR products with SRV/D-T-specific primers Tga3 and Tga4. (C) Agarose gel electrophoresis of first-round PCR products with primers SRVPOLF1 and SRVPOLR1for SRV/Ds (10). (D) Agarose gel electrophoresis of nested PCR products with SRV/D-specific primers SRVPOLF2 and SRVPOLR2 (10). DNA size markers, 100-bp ladder (leftmost lane) and 1-kb ladder (far right lane).

plification was performed as follows: initial denaturation at 95°C for 5 min; 30 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 10 min.

Sensitivity of SRV/D-T-specific PCR. A 2189 bp gag fragment of SRV/D-T was inserted in pCR2.1 vecter (Invitrogen, Tokyo, Japan) to prepare recombinant plasmid for sensitivity test of the PCR. To monitor the sensitivity of the PCR, serial tenfold dilutions of the plasmid DNA (2×10^6 copies to 2 copies) were prepared using 1 µg/µl PCR test-negative genomic DNA from uninfected macaque peripheral blood mononuclear cells (PBMCs). The PCR reaction was performed in duplicate for each dilution, with inclusion of negative, positive, and reagent controls. The nested PCR reaction also was performed in duplicate, by using 2 µl of the first-round PCR reaction as described.

DNA sequencing and analysis. PCR products were purified using an Ultra Clean GelSpin Kit (MO BIO Laboratories, Inc., Solana Beach, Calif.) and directly sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. The cycle sequencing reactions was performed as follows: initial denaturation at 96°C for 1 min; 25 cycles at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Electrophoresis of the samples was performed on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), and sequence analyses performed with GENETYX version 10.1.4 (Genetyx, Tokyo, Japan).

Results

SRV/D-T-specific PCR and sensitivity of PCR. SRV/D-T-specific primers were designed based on the nucleic acid sequences in the *gag* region of SRV/D -1, -2, and -3 and SRV/D-T (Table 1). First-round PCR using the SRV/D-T-specific primer set (Tga1 and Tga2) yielded a 722-bp product from genomic DNA of SRV/D-T infected Raji cells (Fig. 1A); no product was detected from monkey blood cell DNA samples. To increase the sensitivity and specificity, nested PCR was performed using a second primer set (Tga3 and Tga4) to amplify a 381-bp band (Fig. 1B). Products were not obtained from SRV/D-1 or -2 proviral DNA, showing the specificity of the Tga1 through Tga4 PCR primers for SRV/D-T. In contrast, in the first-round PCR, using the primer set for SRV/D subtypes 1 through 5 (SRVPOLF1 and SRVPOLR1) led to amplification of a 446-bp product from SRV/D-1, SRV/D-2, and SRV/D-T (Fig. 1C). In addition, the second round of nested PCR, using primers SRVPOLF2 and SRVPOLR2, led to amplification of a 181-bp fragment from SRV/D-1, SRV/D-2, and SRV/D-T as expected (Fig. 1D).

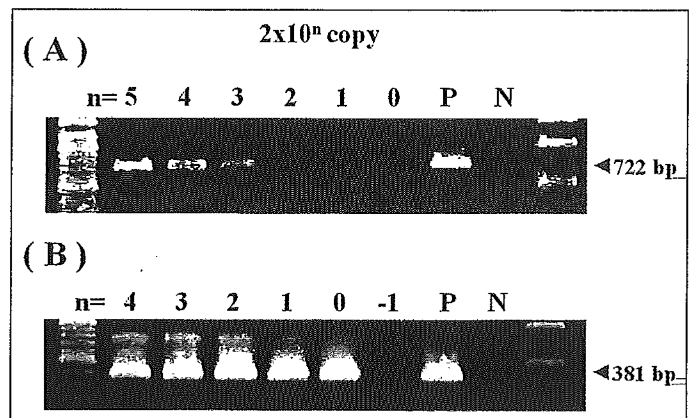

Figure 2. Sensitivity of PCR assays using SRV/D-T-specific primer sets. (A) Agarose gel electrophoresis of first-round PCR products on plasmid template with the Tga1 and Tga2 primers in the presence of 1 µg genomic DNA from macaque blood cells. (B) Agarose gel electrophoresis of nested PCR products with the Tga3 and Tga4 primers.

Using the cloned SRV/D-T *gag* plasmid, we determined the sensitivity of the PCR assay by end-point titration in the presence of carrier DNA. In first-round PCR using Tga1 and Tga2, as few as 2000 copies were detectable (Fig. 2A), whereas nested PCR adding Tga3 and Tga4 could detect as few as two genome copies (Fig. 2B).

Detection of SRV proviral DNA from macaque blood cells by SRV/D-T-specific PCR. SRV/D-T-specific PCR was performed using genomic DNA from whole peripheral blood cells of cynomolgus monkeys infected with SRV/D-T. As negative controls, DNA samples were prepared from blood of uninfected macaques. When genomic DNA from macaques infected with SRV/D-T was tested, the expected target 381-bp product was amplified effectively by nested PCR (Fig. 3A). However, using SRV/D-1 or -2 as template DNA for the nested PCR with these primers yielded no signal, and DNA from uninfected macaque blood lacked amplification of specific bands as well (Fig. 3A). Nested PCR using the SRVPOLF2 and SRVPOLR2 primers amplified a 181-bp fragment from blood-cell DNA from an SRV/D-T-infected macaque (Fig. 3B). Furthermore, the same 181-bp fragment was observed on macaque genomic DNA from uninfected monkeys (Fig. 3B). Because these generic primer sets from the *pol* region of SRV/D might also amplify endogenous type D retroviral sequences present in macaque genomic DNA (22) (Fig. 3B), we tested another primer set, which detects *gag* sequences of multiple SRV/D subtypes (Table 1, Fig. 3C). These results confirmed the specificity of the Tga primer sets for SRV/D-T and demonstrated

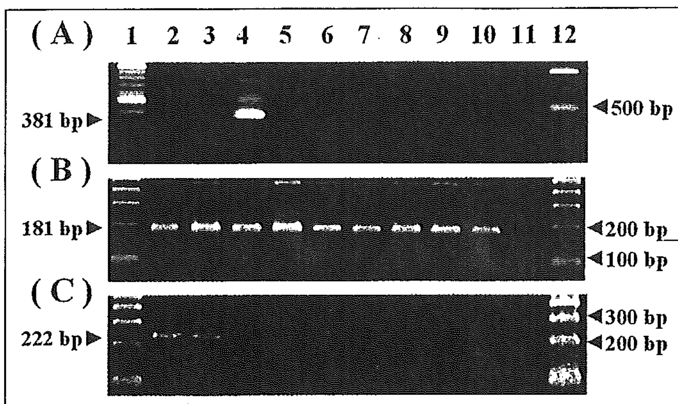

Figure 3. Detection of SRV/D-T signal from blood genomic DNA by nested PCR assay. (A) Tga1–Tga2 and Tga3–Tga4 primer sets were used for nested PCR amplification from blood genomic DNA of the monkey infected with SRV/D-T. (B) SRVPOLF1–SRVPOLR1 and SRVPOLF2–SRVPOLR2 primer sets. (C) WH1–WH3 and RC12–RC15 primer sets. Sources of template DNAs used are as follows: lane 2, Raji cells infected with SRV/D-1; lane 3, Raji cells infected with SRV/D-2; lane 4, blood cells from the monkey infected with SRV/D-T; lanes 5 through 10, blood cells from uninfected monkeys; lane 11, no DNA control. Lane 1, 100-bp ladder; lane 12, 1-kb or 100-bp ladder.

the lack of amplification of any endogenous SRV/D sequences by using these primer sets.

Survey of SRV/D infection in cynomolgus macaques at TPC. Previously, we reported the isolation of SRV/D-T from two cynomolgus monkeys in our breeding colony. However, it was unknown whether this subtype was prevalent in the TPC colony or if it were only a rare occurrence. To this end, EDTA-treated blood samples from 60 animals in the SPF colony and 49 retired breeders from the conventional colony were collected for genomic DNA preparation. We used the SRV/D-T-specific primer set and several other primer sets that detect other SRV/D subtypes (WH1-WH3 and RC12-RC13 for SRV/D-1 and -3 and WH1-WH3 and RC14-RC15 for SRV/D-2) (Table 1). Because the generic SRVPOLF2 and SRVPOLR2 primers appear to recognize endogenous SRV/D-related sequences (Fig. 3B), we did not use this primer set for direct detection of SRV/D from macaque blood DNA.

Using primers specific for SRV/D-1 and -3 or SRV/D-2, we detected no PCR signals for SRV/D in SPF or conventional monkeys. Use of the SRV/D-T specific primers also failed to amplify any products from the 60 SPF animals, confirming the SRV/D-free status of this colony. In contrast, SRV/D-T-specific primers amplified products from blood DNA samples from 11 of 49 monkeys (22.5%) from the conventional colony (Table 2). These 381-bp products were subjected to sequence analysis, and the results confirmed that all matched with that of SRV/D-T (data not shown). These results indicate that SRV/D subtypes 1 through 3 are not present in the TPC colony and that SRV/D-T is probably the only subtype. Regarding the SRV/D serology of the PCR-positive monkeys, three were seropositive, two were Western blot-indeterminate, and six were seronegative (10).

Discussion

The exogenous simian type D retroviruses occur in wild monkeys, are prevalent in captive populations of Asian macaque monkeys, and can cause immunodeficiency with anemia, weight loss, and persistent unresponsive diarrhea (2, 3, 5, 12). If macaques infected with SRV/D are used in biomedical experiments, the results may be affected by the animal conditions and especially by the onset of clinical signs of disease including immunodeficiency (9). For these reasons, it is important to eliminate SRV/D infections from captive breeding colonies.

Table 2. PCR-based survey of SRV/D infection in specific pathogen-free and conventional colonies at Tsukuba Primate Center

	Positive for			
	SRV/D-T	SRV/D-1, -2, -3	Negative	Total tests
SPF	0	0	60	60
Conventional	11	0	38	49

The blood DNAs from 60 cynomolgus monkeys in the SPF breeding colony and 49 animals in the conventional breeding colony were prepared as described in the Materials and Methods. The Tga1–Tga2 and Tga3–Tga4 primer sets were used to detect SRV/D-T sequences, WH1–WH3 and RC12–RC13 primer sets were used for SRV/D-1 and -3, and WH1–WH3 and RC14–RC15 primer sets used for SRV/D-2 (8).

Since we established our indoor, closed cynomolgus monkey breeding colony, we have conducted periodical health examinations of all monkeys, including serum biochemistry and microbiological testing, every 2 years. Preliminary monitoring of SRV/D infection in our cynomolgus breeding colony indicated that approximately 20% of the animals were seropositive. We recently isolated a novel SRV/D subtype from two seropositive cynomolgus macaques in our conventional breeding colony that exhibited clinical signs of SRV/D infection. The *gag* region sequence of SRV/D-T differed from known SRV/D subtypes -1, -2, -3, and -5. Because some monkeys are inapparent virus carriers without detectable antibody responses (7), it is necessary to perform both serological testing and detection of virus presence in order to eradicate this virus from a breeding colony. To detect SRV/D-T specifically, we designed SRV/D-T-specific PCR primer sets based on the aligned gag sequences of SRV/D -1, -2, and -3 and SRV/D-T (Table 1). Using these specific primer sets, we were able to detect SRV/D-T-specific 722-bp and 381-bp products in first-round and nested PCR assays, respectively (Fig. 1). Moreover, the nested PCR for SRV/D-T was sensitive enough to detect as few as two copies of the viral genome (Fig. 2B). In order to simplify the testing procedure, we introduced the method of preparing genomic DNA directly from whole blood treated with EDTA. Using such genomic DNA as template for nested PCR, we were able to detect SRV/D-T directly in DNA prepared from blood cells of infected monkeys without having to purify PBMCs (Fig. 3A).

Although we isolated SRV/D-T from two cynomolgus monkeys in our breeding colony, it was unknown whether this subtype is prevalent at TPC. In order to investigate the subtypes of SRV/D present in our cynomolgus monkey colony, we used our SRV/D-T-specific primer sets and published primers that detect other SRV/D subtypes (8). Genomic DNA samples from the conventional and SPF monkey colonies were surveyed. The PCR-positive rate for SRV/D infection in retired breeders from the conventional colony was 22.4% (11 of 49), whereas no positive monkeys were detected in the SPF colony. Primer sets that simultaneously detect multiple SRV/D subtypes failed to identify SRV/D signals in either colony. Furthermore, 12 SRV/D isolates from captive-bred cynomolgus macaques housed in two separate buildings at TPC also were demonstrated to be SRV/D-T by sequence analysis of PCR products (data not shown). Therefore, we conclude that SRV/D-T is the major subtype of SRV/D present in the TPC conventional breeding colony.

There are several possibilities for the dominance of subtype SRV/D-T in the TPC colony. First, when the monkeys were imported from Southeast Asian countries, perhaps most were not infected with SRV/D, and only a few were infected with subtype SRV/D-T. This scenario seems unlikely because of a report of the isolation of SRV/D-2 from Indonesian cynomolgus monkeys (16). Therefore, we expect that the Indonesian cynomolgus monkeys in our breeding colony would have been infected with both SRV/D-2 and SRV/D-T. A second possibility is that particular subtypes of SRV/D endemic to monkeys in each country were introduced into the colony through importation. If this were the case, we have to consider why only SRV/D-T survived in the colony. Although further virological studies on SRV/D isolates are needed, SRV/D-T may have unique biological characteristics—such as abundant shedding of the virus in body fluids, increased stability of shed virus, higher infectivity, and/or increased replication rate—that allowed it to be readily transmitted within the colony from only a few infected monkeys. Considering the morphology, titer in cell culture, and genome structure of SRV/D-T, this possibility seems unlikely.

As previously described, to establish a large cynomolgus monkey breeding colony, we imported monkeys from the Philippines, Indonesia, and Malaysia between 1979 and 1983. After establishment of a closed breeding colony, periodic health examination with serum banking was conducted every year, and mating records, pedigrees, and daily observation records were all archived. Screening of banked sera dating from the original quarantine period may help identify the origin of SRV/D-T. Alternatively, it may be necessary to screen wild monkeys from each country of Southeast Asia by using our established PCR method to detect SRV/D-T and determine its origin.

Acknowledgments

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Original article

Isolation and characterization of a new simian retrovirus type D subtype from monkeys at the Tsukuba Primate Center, Japan

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Abstract

Exogenous type D simian retroviruses (SRV/D) are prevalent in captive and feral populations of various macaque monkeys. Thus far, five subtypes of SRV/Ds have been reported, three of which (SRV-1, -2 and -3) have been molecularly characterized. Two SRV/D strains (N27 and T150) were isolated from seropositive cynomolgus macaques at the Tsukuba Primate Center (TPC) in Japan, showing clinical signs of SRV/D infection, including anemia and persistent unresponsive diarrhea. Electron microscopy demonstrated that both SRV/D isolates have a virion morphology typical of type D retrovirus. The SRV/D N27 and T150 isolates were essentially the same based on sequence analysis. From homology analysis of the entire *gag* sequence, the N27 isolate is closely related to the other known SRV/Ds but is distinct from the three molecularly characterized SRV/Ds. Thus, we have tentatively designated the N27 and T150 viruses isolated from TPC cynomolgus macaques as SRV/D-Tsukuba (SRV/D-T).
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Keywords: Simian retrovirus type D; Cynomolgus macaque; Virus isolation; *gag* sequence

1. Introduction

Infection with simian retrovirus type D (SRV/D) is prevalent in wild as well as colony-born macaques [1–3]. Different isolates are associated with different macaque species at different primate research centers [2]. Thus far, five subtypes have been identified based on virus neutralization [2–5], of which three (SRV-1, SRV-2, and Mason–Pfizer monkey virus; MPMV; SRV-3) have been molecularly cloned and sequenced [6–8]. SRV-4 was isolated from a cynomolgus macaque at University of California, Davis, but its molecular characterization has not been reported. Recently, the partial *gag-prt* sequence of SRV-5 was determined [9]. Also, SRV/D-6 was isolated from a Hanuman langur and its partial sequence was reported [10,11].

SRV/D has been identified as one of the causes of simian acquired immunodeficiency syndrome (SAIDS), a naturally occurring immunosuppressive and sometimes fatal disease of macaques. Animals suffering from advanced SAIDS may exhibit lymphadenopathy, splenomegaly, anemia, lymphoid depletion, bone marrow hyperplasia, weight loss, persistent unresponsive diarrhea, chronic opportunistic infections and malignant neoplasia [12].

Macaques are needed for a variety of biomedical studies such as preclinical studies and/or safety testing of vectors for gene therapy in which immunosuppressive treatments may result in an increase in the viral load of SRV/D. Also, macaque models provide an opportunity to develop vaccines against simian immunodeficiency virus (SIV), which induces a disease similar to human AIDS. Thus, the detection and eradi-

Abbreviations: CPE, cytopathic effect; PBMCs, peripheral blood mononuclear cells; SAIDS, simian acquired immunodeficiency syndrome; SRV/D, simian retrovirus type D; TPC, Tsukuba Primate Center.

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cation of SRV/D infection in macaque breeding colonies is of great importance.

The prevalence of SRV/D infections in the cynomolgus macaque breeding colony at Tsukuba Primate Center (TPC) has been estimated by serological testing. However, whether the monkeys are really infected with SRV/D or not and the subtype of the SRV/D, if any, are not known. In this study, we report the results of analysis of SRV/D viruses isolated from two seropositive cynomolgus monkeys at TPC that exhibited the characteristic clinical signs of SRV/D infection.

2. Materials and methods

2.1. Animals

The two female cynomolgus monkeys (*Macaca fascicularis*) described in this report (no. 27 and no. 150) were bred and reared at TPC, National Institute of Infectious Diseases. The familial lineages of these two monkeys were unrelated. Although serological testing at 6 months of age indicated that monkey no. 27 was positive for SRV/D; she exhibited normal weight gain and growth up to 2 years of age. At this time, body weight and red blood cell (RBC) counts decreased, respectively, from 1.90 to 1.68 kg and from 590 to 166 × 10^7 per ml in the next 6 months. When blood was collected for SRV/D virus isolation at 2.5 years of age, this monkey had occasional diarrhea. Although monkey no. 150 was known to be seropositive to SRV/D at 6 years of age, she remained apparently healthy until 7 years of age. Over a 3-month period, body weight decreased from 3.26 to 2.88 kg and she developed unresponsive diarrhea. RBC counts also decreased from 611 to 86 × 10^7 per ml during this period. At the time peripheral blood samples were collected from these monkeys for serology and virus isolation, both monkeys were positive for SRV/D by Western blotting analysis.

2.2. Virus isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from monkeys no. 27 and no. 150 by Ficoll gradient centrifugation, washed in phosphate-buffered saline (PBS), and aliquoted at 10^6 cells per ml. Approximately 5 × 10^5 PBMCs were cocultured with 2 × 10^5 Raji cells (a Burkitt's lymphoma B-cell line) in complete medium which consisted of RPMI-1640 supplemented with 10% fetal calf serum, 2-mercapto-ethanol (50 μM), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), concanavalin A (10 μg/ml), and recombinant interleukin-2 (200 U/ml) (Shionogi and Co., Ltd., Osaka, Japan). Viral cytopathic effect (CPE) and reverse transcriptase (RT) activity were monitored during co-cultivation.

2.3. Electron microscopic analysis

When CPE became widespread in Raji cells co-cultivated with PBMCs from a monkey No. 27, cells were fixed in ice-cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.5) and washed with 0.1 M cacodylate buffer containing 0.1 M sucrose. The fixed cells were embedded in Epon 812 resin and ultra-thin sections were prepared, stained with lead citrate/uranium acetate, and analyzed by electron microscope (Hitachi H7600).

2.4. Western blotting

Antibodies to SRV/D were screened by Western blotting using SRV/D-2 antigen supplied by Bio-Reliance (MD, USA). The criterion for a positive reaction was detection of two or more virion-specific bands (i.e. gag and env).

2.5. Polymerase chain reaction

DNA from SRV/D-infected Raji cells was extracted using NucleoSpin Blood Kit (MACHEREY-NAGEL, Duren, Germany). The *gag* region (222 bp) was amplified by nested PCR using primers that detect SRV/D-1, -2, and -3 (WH1, WH3 and RC12–15) [13]. Other *gag* primer sets (p27F1, p27R1 and p27F2, p27R2) that detect part of the p27 region of SRV/D-1 to -5 were also used [14].

The entire *gag* region was amplified by PCR using primers gast4, 5′-AGCGAAAGTACATTGTCTTA-3′ and RR111, 5′-TCATTGTCGACAACCCCTGGA-3′. PCR was performed with 500 ng of genomic DNA, 20 pmol of each primer, 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM dNTPs and 2.5 units of Takara LA *Taq* polymerase (Takara, Ohtsu, Japan) in a total volume of 50 μl. PCR was performed in a Peltier Thermal Cycler Model PTC-200 (MJ Research, Tokyo, Japan). Amplification was performed as follows: one cycle at 95 °C for 4 min; 30 cycles at 94 °C for 1 min; 54 °C for 1 min and 72 °C for 4 min; and one cycle at 72 °C for 10 min. Amplified PCR products were visualized by electrophoresis in 0.5% agarose gels stained with 0.5 mg/ml ethidium bromide under ultraviolet transillumination.

2.6. DNA sequencing

PCR products were purified using an Ultra Clean GelSpin Kit (MO BIO, CA, USA) and were directly sequenced using the BigDye Terminator Ver. 1.1 Cycle sequencing Kit (Applied Biosystems, Tokyo, Japan), according to the manufacturer's protocol. The templates were subjected to one cycle at 96 °C for 1 min, 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Electrophoresis was performed on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

2.7. Analysis and alignment of nuculeotide sequences and amino acid sequences

Analysis and alignment of nuculeotide sequences and amino acid sequences were done with GENETYX Ver. 10.1.4

(genetyx@SDC.co.jp). Gaps introduced for optimal alignment were not considered informative and were not included in the analyses. Distances were estimated by Kimura's two-parameter method for nucleotide substitution [15]. The phylogenetic trees were constructed by the neighbor-joining (NJ) method [16]. The GenBank accession numbers of the sequences used for comparison were SRV/D-1 (M11841) [6], SRV/D-2 (AF126467) [17], SRV/D-3 (M12349) [7], BaEV (D10032) [18], SMRV-H (M23385) [19], GALV (M26927) [20], SFV (X54482) [21], HTLV-1 (L03561) [22], HTLV-2 (NC-001488) [23], STLV-2 (NC-001815) [24] and RSV (J02342) [25]. The *gag* sequence from the strain SRV/D N27 (SRV/D-Tsukuba, SRV/D-T) has been deposited in GenBank (DDBJ) under accession number AB181392.

3. Results

3.1. Isolation of SRV/D from cynomolgus monkeys

When PBMCs of the two monkeys that developed SAIDS with SRV/D were co-cultivated with Raji cells, typical SRV/D CPE including syncytia was observed after 4 days (Fig. 1A). RT activity was also detected in the culture supernatant, and Raji cells with syncytia were positive by indirect immunofluorescent antibody assay using anti-SRV/D-2 serum. When these infected Raji cells were subjected to electron microscopic analysis, typical A-particle-like virions (90 nm in diameter) were observed in the cytoplasm and mature type D particles (120 nm in diameter) were observed outside of the cells (Figs. 1C,D) [4]. SRV/Ds isolated from monkeys No. 27 and No. 150 were tentatively designated SRV/D N27 and SRV/D T150, respectively.

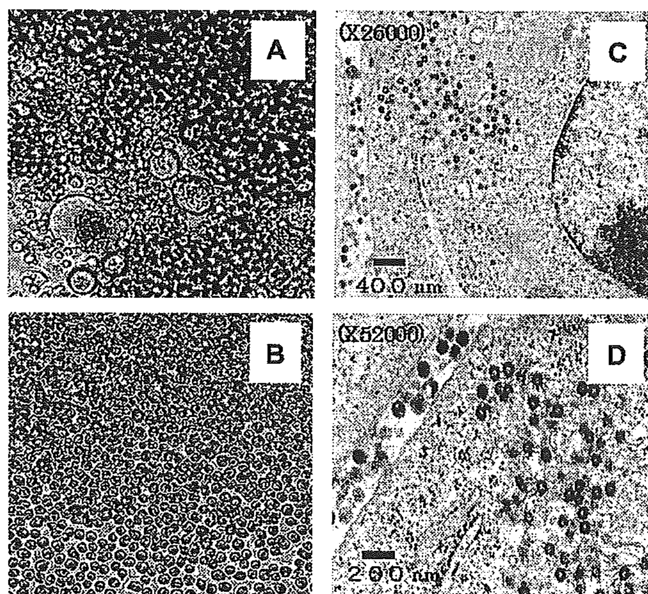

Fig. 1. CPE and virion morphology of Raji cells infected with SRV/D isolate from cynomolgus monkey No. 27. A: Infected Raji cells with large syncytia (×100). B: Uninfected Raji cells (×100). C: Electron micrograph of infected Raji cells (×26,000). D: Higher magnification electron micrograph of cytoplasmic 90-nm type A retrovirus particles and extracellular mature type D retroviruses.

3.2. Characterization of isolated SRV/D from TPC

The genomic DNA from Raji cells infected with the N27 and T150 isolates were subjected to PCR analysis to amplify the *gag* region, using primer sets (WH1, WH3 and RC12–15) which detect only SRV/D-1, -2 and -3 [13]. These primers give a strong signal for these three viruses at both the first and second rounds of the nested PCR, but not for SRV/D-4 or -5 (N.W. Lerche, California National Primate Research Center, USA, personal communication). No amplified band was observed at the first amplification using WH1, WH3 primer sets on the genomic DNA of Raji cells infected with the N27 or T150 isolates. However, a 232-bp band was detected by nested PCR with the RC12–15 primers (data not shown). Furthermore, a 461-bp and a 400-bp band were observed on first and second rounds of PCR, respectively, using published primer sets (p27F1, p27R1 and p27F2, p27R2) [14]. These primers detect the p27 *gag* region of SRV/D-1 to -5.

PCR results suggested that the N27 and T150 isolates are not SRV/D-4 or -5 but are closely related to SRV/D-1 to -5. Thus, we tentatively named the N27 and T150 isolates SRV/D-T. In order to characterize the SRV/D-T virus further, we sequenced the entire *gag* region of the N27 strain.

3.3. Comparison of the gag nucleotide sequence of SRV/D-Tsukuba with other SRV/D subtypes

Genomic DNA of N27 strain-infected Raji cells was prepared, and a 2189-bp fragment from the *gag* region of the integrated proviral DNA was amplified by PCR using the *gag* primer sets described in Section 2. Analysis of the amplified sequence revealed one large open-reading frame (ORF) (1980 bp; 659 aa). The sequence of the 1980-bp *gag* ORF of the N27 isolate was compared with the corresponding regions of SRV/D-1, -2 and -3 (Fig. 2). The nucleotide homology between the Tsukuba N27 isolate and other SRV/D strains varied from 78.5% to 81.1%, whereas the homology between SRV-1 and SRV-3 was 92.8% and the homology between SRV-1, -3 and SRV-2 was 79.2% and 79.6%, respectively (Table 1). Comparison of deduced amino acid sequences of the N27 isolate and other SRV/Ds gave similar results, suggesting that the N27 isolate is closely related to, but distinct from, the other known SRV/Ds (Table 1).

Since only a part of the *gag-prt* region of SRV/D-5 has been published (AF252389) [9], the corresponding region of the N27 strain (629 bp) was compared with that of SRV/Ds. Nucleic acid sequence homology between the N27 strain and SRV/D-1, -2, -3, and -5 in the *gag-prt* region was 83.5%, 83.0%, 82.8% and 84.1%, whereas the homology between N27 isolate and T150 strain was 99.7%.

In the *gag* region, five post-translational proteolytic products (p10, p12, p27, p14 and p4) have been reported for SRV/D-1, -2 and -3 [26,27]. Alignment of the deduced amino acid sequence of the N27 strain revealed that the probable proteolytic cleavage sites for each polypeptide are identical

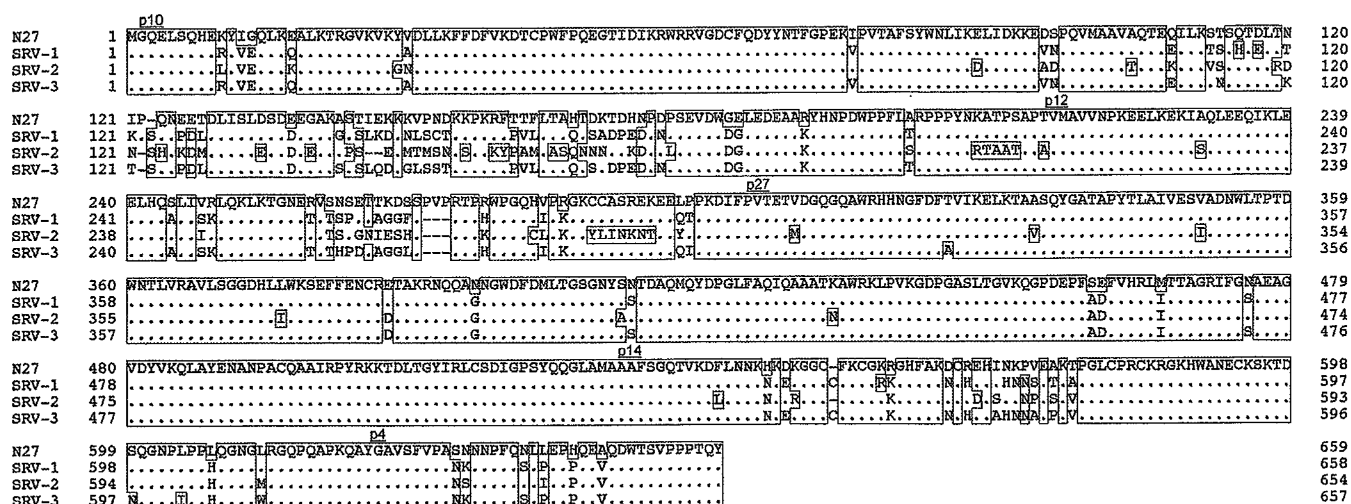

Fig. 2. Alignment of amino acid sequences in the *gag* region of the SRV/D N27 isolate and SRV/D-1, -2, -3. The probable proteolytic cleavage sites for generation of the p10, p12, p27, p14, and p4 polypeptides are indicated.

Table 1
Percent homology of nucleic acid and amino acid sequences of the entire SRV/D *gag* region

Sequence	N27 vs SRV-1	N27 vs SRV-2	N27 vs SRV-3	SRV-1 vs SRV-2	SRV-1 vs SRV-3	SRV-2 vs SRV-3
Nucleic acid	81.1	78.5	80.6	79.2	92.8	79.6
Amino acid	86.2	82.9	85.9	84.0	96.2	84.3

Table 2
Percent homology of nucleic acid sequences of post-translational proteolytic products in *gag* genes

Region	N27 vs SRV-1	N27 vs SRV-2	N27 vs SRV-3	SRV-1 vs SRV-2	SRV-1 vs SRV-3	SRV-2 vs SRV-3
p10	92.0	91.0	92.0	92.0	100.0	92.0
p12	76.7	67.4	75.6	67.5	95.2	66.3
p27	97.4	95.2	96.5	96.0	99.1	96.0
p14	85.3	89.4	82.1	84.2	91.6	84.2
p4	83.3	88.9	83.3	88.9	100.0	88.9

(Fig. 2). Table 2 shows the high homology of p10 and p27 (91.0–100%) and the intermediate homology of p12 and p14 (66.3–95.2%) in all moleculary characterized SRV/Ds, including the N27 isolate. It is interesting that while the N27 isolate has greater homology to SRV/D-1 and -3 in the p10, p12 and p27 regions, it had significantly greater homology to SRV/D-2 in the p14 and p4 regions.

3.4. Phylogenetic analyses

The nucleotide sequence of the entire *gag* gene was analyzed to evaluate the phylogenetic relationship of the N27 strain with other known SRVs and related retroviruses based on sequences available from the GenBank database.

These analyses placed the new Tsukuba virus firmly in the SRV/D group (Fig. 3). While SRV/D-T was more closely related to SRV/D-2 than to SRV/D-1 and -3, the branch length separating N27 and SRV/D-2 was greater than that separating SRV/D subtypes 1 and 3. These, together with the varying degree of homology of individual *gag* derived proteins, indicate that the Tsukuba isolate represents a new subtype of SRV/D (Fig. 3).

4. Discussion

According to published observations on clinical findings, the outcomes of SRV/D infection can be divided into three categories: (a) no apparent clinical signs of disease or viremia with a high antibody titer, (b) low viremia and a transient antibody response with or without chronic disease, and (c) a high viremic state with rapid disease progression and no antibody response [28,29]. Cynomolgus monkeys from which SRV/D-T was isolated were seropositive for SRV/D but had no clinical signs of disease for several years. Thus, the two monkeys would belong to category (a). Based upon periodical health examinations and daily observations of these two monkeys, they were suspected of experiencing the onset of SAIDS (having anemia, weight loss, and persistent unresponsive diarrhea). At this time, blood samples were collected for virus isolation.

We are convinced that the virus isolates derived from the monkey PBMCs are a subtype of SRV/D based on the following evidence: (1) co-cultivated Raji cells showed typical SRV/D CPE, including syncytia (Fig. 1A); (2) RT activity was detected in the culture supernatant only after co-cultivation; (3) infected Raji cells were positive using anti-

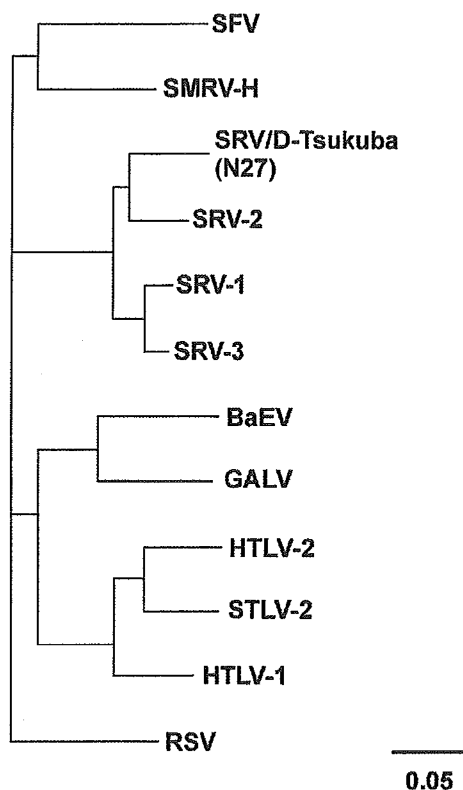

Fig. 3. Phylogenic analysis of SRV/D-T (N27 strain) gag sequence and other retrovirus *gag* genes. The phylogenetic tree was constructed by the NJ method based on the entire 1980-bp *gag* gene of the SRV/D N27 isolates. Branch lengths are proportional to estimated number of substitutions per site, which represent the evolutional distance.

SRV/D-2 serum in an indirect fluorescent assay; and (4) electron microscopic analysis of infected cells clearly demonstrated the presence of virions having a type D morphology (Figs. 1C,D). Therefore, we have designated the two SRV/D isolates from cynomolgus monkeys SRV/D-T.

The entire *gag* gene nucleotide sequence and deduced amino acid sequence of the N27 isolate had high homology to other known SRVs (Fig. 2, Table 1), and this close similarity extended to individual post-translational proteolytic products (p10, p12, p27, p14 and p4; Fig. 2) [26,27]. The highest homology was observed in the p10 and p27 regions, while the homology in p12 and p14 was somewhat lower (Table 2). Also, phylogenetic analyses confirmed that the N27 strain is closely related to, but distinct from, the other three known SRV/Ds (Fig. 3).

In relation to SRV/D-5, we compared the *gag* sequence of our N27 isolate (629 bp) with the published *gag-prt* region of SRV/D-5 [9]. Homology was 84.1%, whereas homology between the N27 and T150 isolates was 99.7%. Furthermore, the homology between the N27 and T150 isolates and SRV/D-1, -2 and -3 was in a range of 82.8–83.5. Accordingly, the N27 and T150 isolates are essentially the same and closely related to, but distinct from, SRV/D-1, -2, -3 and -5. Regarding SRV-6, the Indian Hanuman langur from which this virus was isolated belongs to a completely different genus and its resident area is different from that of the TPC cynomolgus monkeys imported 25 years ago [30]. Therefore, even though sequences are not available for direct comparison, we do not think our SRV/D isolates are likely to be identical to SRV-6.

The relationship of the N27 and T150 isolates to SRV/D-4 is less clear. SRV/D-4 is known only from a single isolate obtained from a cynomolgus monkey in California [13]. Only when sequence data become available for this isolate will we be able to confirm this. Although we detected 461- and 400-bp PCR products using published primer sets [14] which detect the p27 region of SRV/D-1 to -5, a 232-bp band was also detected upon nested PCR with primers that amplify SRV/D-1, -2 and -3 [13] but do not amplify SRV/D-4 and -5 (N.W. Lerche, California National Primate Research Center, USA, personal communication). Thus, we do not believe that our SRV/D isolate is the same as SRV/D-4 or -5.

Reconciling the results of the PCR detection and morphological study, the N27 and T150 isolates appear to be more closely related to SRV/D-2 and SRV/D-1 and -3 than to SRV/D-4 or -5. Therefore, we have tentatively designated the N27 and T150 isolates as strain SRV/D-T. Complete genome sequence analysis and comparison of all the SRV/D subtypes will be necessary to definitively classify these viruses.

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ORIGINAL PAPER

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Identification of the MHC class I *B* locus in cynomolgus monkeys

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Abstract By determining the nucleotide sequences of more than 700 cDNA clones isolated from 16 cynomolgus monkeys, we identified 26 *Mafa-B* alleles. In addition, nine sequences with similarity to *Mamu-I* alleles were identified. Since multiple *Mafa-B* alleles were found in each individual, it was strongly suggested that the cynomolgus MHC class I *B* locus might be duplicated and that the *Mafa-I* locus was derived from the *B* locus by gene duplication, as in the case of the *Mamu-I* locus of rhesus monkeys.

Keywords Cynomolgus · MHC · *Mafa* · Allele

Introduction

It is well established that $CD8^+$ T-cell activation is triggered through recognition of the MHC class I molecule loaded with an antigenic peptide by an antigen-specific T-cell receptor. The MHC molecules of the mammals including primates are known to influence the outcome of many diseases such as infectious diseases, cancer, and metabolic disorders. HLA class I genes are divided into three different categories, classical (*HLA-A*, *-B*, and *-C*), non-classical (*HLA-E*, *-F*, and *-G*), and pseudogene (*HLA-H*, *-J*, *-K*, and *-L*), according to their degree of polymorphism and cell surface expression, and the presence of orthologues of the human *HLA-A*, *-B*, *-E*, *-F*, and *-G* genes were identified in several species of the Old World monkeys (Alvarez et al. 1997; Boyson et al. 1996a,b; Evans et al. 2000; Lafont et al. 2004; Otting and Bontrop 1993; Prilliman et al. 1996; Sidebottom et al. 2001; Uda et al. 2004). Cynomolgus monkeys as well as rhesus monkeys are preferentially used for biomedical research; however, cynomolgus MHC class I was not extensively studied compared with those in rhesus monkeys. We have previously reported the nucleotide sequences of cynomolgus MHC class I *A* locus and have shown that at least 14 *Mafa-A* alleles were present in cynomolgus monkeys (Uda et al. 2004). Although the MHC class I *B* locus is the most polymorphic MHC locus in primates, little information is available concerning the MHC class I *B* locus of cynomolgus monkeys. In this study, therefore, we have expanded our analysis on cynomolgus MHC class I genes and identified 26 *B* locus alleles by analyzing 16 monkeys. We have also found the presence of a novel locus that is very similar to MHC class I *I* locus recently identified in rhesus monkeys.

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Materials and methods

Animals

All cynomolgus monkeys were raised and reared in the Tsukuba Primate Center for Medical Science, the National Institute of Infectious Diseases (NIID). Both genders were involved, and the cynomolgus monkeys were between 5 and 24 years old. This study was conducted in accordance with the Guides for Animal Experiments Performed at the NIID.

RT-PCR and nucleotide sequencing

Preparation of mRNA from peripheral blood mononuclear cells (PBMC) and RT-PCR were performed as described before (Uda et al. 2004). Primers used in this study are listed in Table 1. 5′ MBS and 3′ MBS primers designed to amplify the gene products of the rhesus MHC class I *B*