必携 今日の生殖医療

§ 7　ART のラボ手技

49. 精液中 HIV の完全除去法

49. Semen processing method for complete elimination of HIV-1

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Key Words　精液，HIV，体外受精，改良 Swim up 法，超高感度 PCR

治療の進歩にともないエイズ死亡率が減少し，HIV 陽性の夫を持つ夫婦の挙児希望が増えている．Swim up 法により精液中の HIV 量を減らすことが可能となり，EU を中心に Swim up 法による人工授精が広まりつつあるが，ウィルス除去が完全ではなく安全性に疑問が残る．われわれは改良 Swim up 法により精液からの HIV 完全除去法を開発し，超高感度 PCR でウィルスの完全除去を確認した．現在 HIV 除去精子を用いた体外受精や顕微受精を実施し，良好な成績をあげている．

背　　景

AIDS が死の病であったとき，HIV 陽性男性と陰性女性の夫婦が子どもを希望しても妻に 2 次感染させる可能性があり，挙児をあきらめるよう医療従事者は指導してきた．しかし1996年にプロテアーゼ阻害剤（PI）を含む多剤併用療法（Highly Active Antiretroviral Treatment：HAART）が導入されて以来，AIDS 死亡率は以前の20%以下まで減少し，HIV/AIDS はコントロール可能な慢性感染症になりつつある．長期生存が可能になった HIV 感染者の間で，結婚して子が欲しいと願う患者が増えている．しかし，夫婦間で男性のみが HIV 感染者の場合，コンドームを使わない性交渉は妻に二次感染する危険があるため，挙児をあきらめるケースが多かった．

こうしたなか，人工授精による二次感染防止の試みがイタリアを中心に EU 各地で行われている．しかし，ウィルス除去に関して十分な安全性が確認されておらず，アメリカ CDC（Centers for Disease Control and prevention）は今でも禁止勧告を解除していない．HIV に感染した乳幼児の予後はいまだに不良で，抗 HIV 薬の催奇形性の問題もある．筆者らは母も子も感染させないために HIV 感染者の精液から HIV を完全に除去し，100%安全に子ができる方法を開発した[1]．

精液中の HIV 存在様式（図 1）

精液には精漿の HIV-RNA のほかに精子に付着した HIV-RNA が存在し，精液中の単核球には proviral DNA が検出されている．精液中の HIV で感染力が最も強いのは感染細胞から作られる HIV-RNA である．抗 HIV 療法で血液中のウィルス量（Viral Load：VL）が検出限界以下になった場合

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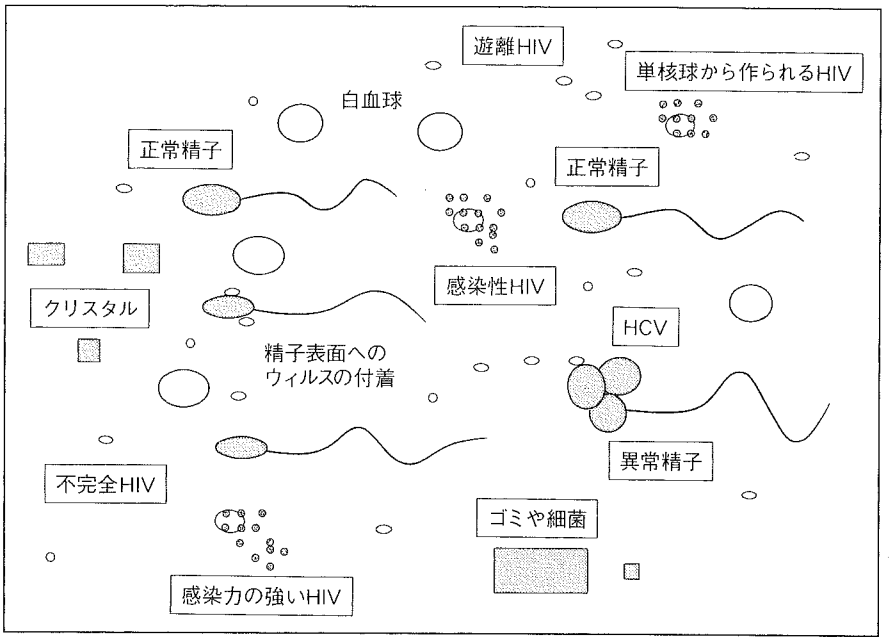

図1　精液中の HIV 存在形式

表1　各方法による女性の2次感染確率と課題

性　交　渉	毎年約4.8% ずつ感染 血中 HIV 量と感染確率は相関 精子の数が少なく、運動率が低下す妊娠率が低い
従来の Swim up 法での人工授精	1/2000以下だが 0 ではない 回収精子数が少ないと妊娠確率が低い
われわれの改良法による体外受精	理論上は100%安全

でも，精液の単核球中の HIV proviral DNA は残存し，精液から二次感染するリスクは残るのでコンドームの使用は必要になる[2]．

受精法によるリスク　(表1)

1．性交渉による二次感染の危険性

異性間性交渉による HIV の二次感染率は1回あたり約0.15%（1~2/1000）といわれるが，AIDS が進行している場合は5/1000，安定している場合は0.7/1000程度である[3]．しかしクラミジア感染症などの性感染症があると HIV に3～5倍感染しやすくなり，HIV に感染するとほかの性感染症に感染しやすくなる．

Quinn らは血中 VL が多いほど性交渉による二次感染の確立は高くなり，VL が400以下では二次感染が少ないと報告している[4]が，調査人数が少なく VL をどれだけ下げると安全かは不明である．花房らの検討では血中 VL と精液中 VL には相関が認められ[5]，抗 HIV 療法で血中 VL が少なくなると，精液中の VL も少なくなるが，どれだけ減らすと二次感染率が低くなるかは正確には検討されていないし，調査は倫理面からも実施困難である．

2．排卵日のみの性交渉による二次感染の危険性

HIV 陽性の夫が排卵日だけコンドームを使用し

ない場合では毎年約4.8%ずつ妻に感染する[6]．

不妊症の Swim up 法による精子除去とその問題点

HIV 陽性者の無処理の精液を用いた人工授精では約3.5%が二次感染した[7]．それに対し，1992年，Semprini らは不妊症治療の swim up 法を応用して精子処理法を行った．精液中の HIV は精漿中の RNA や単核球中の proviral DNA の形で存在する．精液を遠心分離し，swim up によって HIV が検出感度以下の精子を得た．彼らは1997年までに2,000回以上人工授精を行い，二次感染がないと報告している[8]．しかし，彼らの方法は回収精子で HIV RNA 量が800copies/mL 以下としか確認しておらず，ウィルス除去が不十分で安全性に疑問がもたれてきた．

改良 Swim up 法（不妊症 Swim up 法との相違点）（図2）

その後，より高いウィルス除去率を得るために Swim up 法も研究者によって改良が加えられた．花房らや Pasquier らは精液中の HIV を検出限界以下（50copies/mL）にできたと報告した[5][9]が，その後 nested PCR を用いた検出感度の高い方法を用いた検査では完全除去はできないとの報告が多い[10]．不妊症で行われている従来の方法は HIV 除去が不十分であるうえ，単に検出限界以下というだけでは完全に HIV が除去されたかどうかを確認できない．筆者らは共同研究者の兼子が開発した改良 swim up 法により精液処理を行い，慶應微生物の加藤が開発した 1 copy の HIV を検出する超高感度 PCR を用いて回収精子に HIV が 1 copy もないことを確認した[1]．

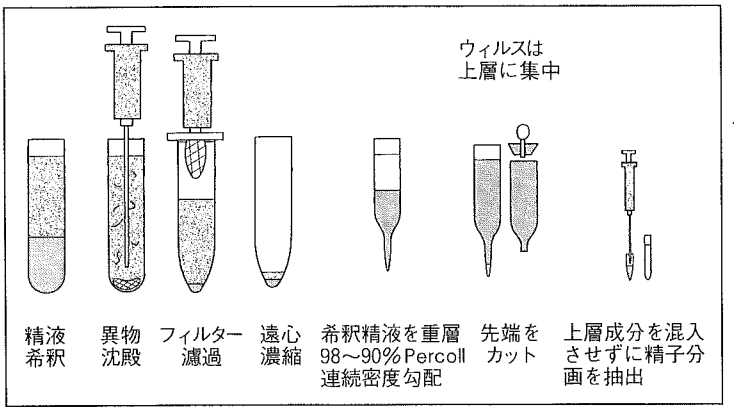

図2-a　精液から HIV 除去を目的とした方法の開発

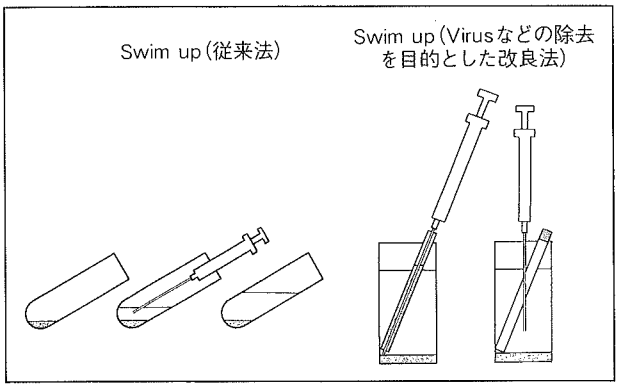

図2-b　Swim up の相違

1．精液希釈と夾雑物除去

精液には前立腺や精嚢腺の分泌物・金属結晶のほかに，尿道通過時の雑菌や下着の繊維などが混入している．そのまま分離液に重層して遠心すると夾雑物が底の精子分画にHIVを巻き込む危険性がある．筆者らは遠心前に精液を培養液で2倍に希釈して室温で20分静置後に上層の精子浮遊液を回収し，フィルターを使ってろ過して遠心分離した．

2．ウィルス分離液と濃度設定の選択

HIV分離液として，Semprini らは1層のみのリンパ球分離液，ほかのグループらは45%と90%の2層のPure Ceptionを用いて，400g，30分間遠心分離した．層を増やすほどウィルス除去率は高まるが精子の回収率が悪くなる．パコールにはエンドトキシン残存問題があり，最近ではPure Ceptionが一般的だが，Pure CeptionによるHIV除去効率はパコールより低い[11]．筆者らは2000年以後，98%パコールを用いた連続密度勾配により，ウィルス除去率を$1/10^6$以上に高めた[1]．

3．遠心分離後の精子分画の回収法（図2a）

遠心分離後に，一番底の洗浄精子をどのように回収するかも問題である．遠心分離後，HIVは上層に分布し，単核球は中間層に分布している．上から順に吸引して精子を回収すると管壁を伝わりHIVが混入する危険性がある．筆者らは兼子らが開発した特殊試験管を用いて，最下層の精子分画部分をカットすることで上層成分を完全に遮断して底の精子を回収している．

4．Swim up法の相違（図2b）

遠心後の精子を培養液で洗浄した後に培養液を重層してSwim upを行うと，比重差がなくウィルスを攪拌する危険がある．われわれは精子液を底にガラス管を通じて静置し，運動精子を回収した．

5．精子や卵へのHIVの感染と付着の可能性

HIVが精子や卵に感染するか否かも議論がある．精子や卵は未分化細胞で，CD4，CCR5やCXCR4が発現していない．HIVのGP120が精子表面の糖脂質に付着し，容量依存性であるとの報告があり[12]，Swim up法で回収した精子にHIVが付着しているか否かが重大な問題だった．

HIV感染成立に必要なCD4蛋白は受精後10週以後にリンパ組織が分化して発現する．体外受精の培養液にウィルスが混入しても卵細胞にウィルスが感染することはない[13]．

6．HIV自体の感染性

HIVは毎日100億個産生されるが，感染力を持っているウィルスは全体の1/10000以下といわれている[15][16]．われわれは，HIVを培養液の中で培養すると，感染性が1/10以下に低下することを示した[16]．

体外受精と人工授精の比較

体外受精は人工授精に比して二次感染防止の面で優れているが，卵巣過剰刺激症候群など女性への負担が大きい．人工授精でHIVが混入した精子浮遊液を子宮に注入した場合，HIV除去が完全でないと子宮内の細胞に感染する危険性がある．一方，体外受精では精子浮遊液にHIVが混入していたとしても卵には感染せず，HIVの感染性は培養後1/10以下に低下する．さらに培養液を2日目に交換し，受精卵を洗浄すると，たとえ精子液にHIVが混入していても相当減少できる．われわれは図3に示すプロトコールに従って，胚移植前に培養液中のHIVがまったくないことを超高感度PCRで確認して胚移植を実施した．

1．HIV感染者の精子の問題

HIV感染者は精子の数が減少し，奇形率も高く，運動率も低下している場合が多い．Swim up法を用いると精子の回収率が低下し，HIV除去率を高めた方法では人工授精では妊娠しなかったことが

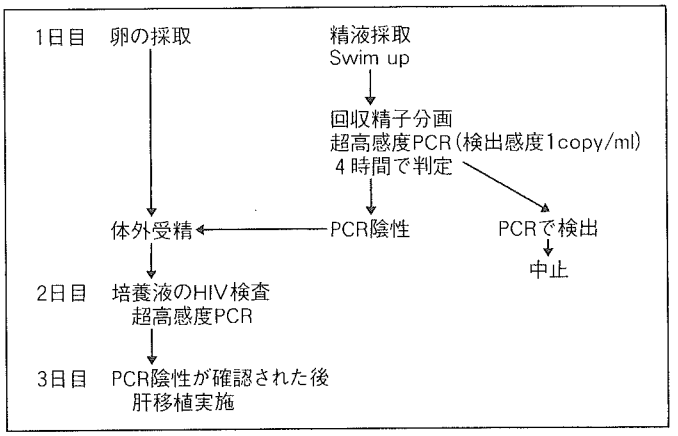

図 3　今回実施した体外受精のプロトコール

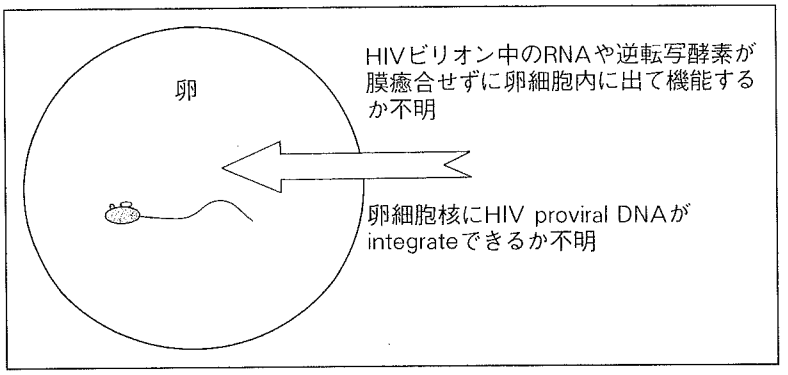

図　4
顕微授精で HIV が付着した精子を卵に強制注入した場合に感染が成立するか否か不明

報告されている[18]．しかし，今後，精子の数が多く，運動率も良い場合には人工授精の適応も必要と思われる．その場合も精子浮遊液の HIV の完全除去の確認が必須と考える．兼子が新たに開発した方法により，精子回収率を高め，ウィルス除去も十分で有れば人工授精の可能性も期待できる．

2．顕微授精

筆者らの方法により，回収精子が少ない場合は顕微授精（intracytoplasmic sperm injection：ICSI）でも二次感染なく妊娠出産できた．図 4 に示すように注入精子に HIV が付着していたり，注入液に HIV が混入していた場合に卵に HIV 感染が成立する可能性は相当低いが，絶対安全とは断言できず，完全除去の確認は必要である．ICSI 自体を問題視する意見もあり今後慎重に対応したい．

3．C 型肝炎の問題

一般に HCV は HIV よりも性交渉による感染リスクは低いとされるが，体外受精による HCV の 2 次感染が報告されており[17]，フランスなどはガイドラインにのっとり夫のウィルス感染検査を必要としている．Pasquier らは Swim up 法で精液中の HCV も除去されることを報告した[9]．

今後の対応

今後，わが国の HIV 感染者の増加にともない，HIV 陽性男性が陰性女性と結婚して子どもを望む機会が増えると予測される．専門機関で精液を処

理し，HIV陰性精子を回収して冷凍保存し，地元の協力産婦人科施設に送って人工授精や体外受精を行うことができれば女性の負担は大幅に減少できる．わが国ではHIV感染者は先進国の中では唯一急増しており，2003年で約15,000人が感染し，2010年には5万人を越えると予測されている．今後，多くの感染者が一般医療機関を受診するケースが増えるので，感染対策は必須である．

おわりに

EUではHIV感染者の人工授精を積極的に行うようになりつつあるが，アメリカCDCはいまだに禁止勧告を解除していない．われわれは不十分なHIV除去法による人工授精で二次感染した女性の相談を受けた．精子浮遊液のHIV RNA, HIV proviral DNAをどれだけ減らせば二次感染が生じないかが不明である以上，完全なHIV除去確認がされていない人工授精は危険である．日本産婦人科学会は2003年からHIV感染者の人工授精は施設の倫理委員会で承認された場合に限定するとの勧告を出した．EUではHIVだけでなくHCV感染症にもガイドラインを作成している国もあり，わが国でも生殖補助医療におけるウィルス感染対策のガイドラインを導入することが必要と考えられる．

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The quantity and diversity of infectious viruses in various tissues of SHIV-infected monkeys at the early and AIDS stages

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Summary. To detect the major sites of viral replication in immunodeficiency virus-infected individuals, we quantified proviral DNA and infectious viruses using quantitative PCR and a plaque assay, respectively, in various tissues of $SHIV_{KU-2}$-infected monkeys in the early and AIDS stages of infection. Compared the quantity of infectious virus among PBMC and the lymphoid tissues, the mesenteric lymph node had the largest number of infectious viruses at the AIDS stage more than at the early stage of infection. These results suggested that the gastrointestinal tract was a major site of viral replication. In the brain, proviral DNA was detected at the early and AIDS stage of infection, but infectious viruses were detected at only the AIDS stage. Moreover, we analyzed the nucleotide sequences of the *env* V3 region in infectious virus clones isolated from each plaque. The viruses in the lymphoid tissues of the monkey that developed AIDS diverged from the inoculated virus and had the same three amino acid substitutions. However, the viruses in the brain were almost identical to the inoculated virus, suggesting that the virus entered the brain early after infection and persisted without replication and genetic diversion until the AIDS stage.

Introduction

HIV-1-related mortality has significantly declined in the United States and other developed countries as a result of the use of effective antiretroviral drugs. Highly active antiretroviral therapy (HAART) has dramatically suppressed the virus load

in the plasma of patients to undetectable levels and retarded the progression of AIDS. However, it is difficult to completely eliminate the virus from the body because of inadequate drug concentration in certain tissues, drug resistance, and persistence of a latently infected pool of cells. Previous studies showed that the frequency of HIV-1-infected cells in lymphoid organs was higher than the frequency in peripheral blood [27, 31, 36]. Moreover, HIV-1-infected cells were detected in various tissues including brain, gut-associated lymphoid tissue and the genital tract [4, 33, 38]. The existence of persistent HIV-1-infected cells or organs could prompt the reappearance of the virus in the infected person even though the virus was once suppressed. Therefore, it is important to identify the reservoir tissues of the virus and the virus characteristics in these sites.

Simian immunodeficiency virus (SIV), which is closely related to HIV, infects monkeys by various routes and induces an AIDS-like disease. Therefore, the SIV-macaque model can provide information about HIV infection in humans [1, 8, 39]. Moreover, some laboratories have constructed chimeric simian-human immunodeficiency viruses (SHIVs), which contain the HIV-1 envelope gene (*env*) within a genetic background of SIVmac239. These SHIVs have been useful in clarifying the role of Env in viral infection, pathogenesis, and the relation between the viruses and the host immune response [11, 14, 21, 28]. Some SHIV strains were found to be pathogenic and cause disease in macaque monkeys. One of them is $SHIV_{KU-2}$, a polyclonal chimeric virus derived from the in vivo passage of SHIV-4, containing the *env* of HIV-1 HXBc2 [15, 24]. $SHIV_{KU-2}$ was reported to cause an AIDS-like disease with encephalitis in macaque monkeys [16, 25].

In this study, to determine the quantity and diversity of viruses in various tissues, we quantified not only proviral DNA but also infectious viruses by quantitative PCR and plaque assay, respectively, and analyzed the hypervariable region (V3) sequences of infectious viruses in peripheral blood mononuclear cells (PBMC), brain, and lymphoid tissues of the $SHIV_{KU-2}$-infected monkeys in the early and AIDS stage of infection. The viral load in the infected individuals has usually been quantified either by the copy number of virus RNA or DNA using PCR [5, 31] or by the end-point-dilution of cell cultures [13, 36]. However, the former method did not differentiate between infectious and non-infectious viruses. Although the latter method is useful for quantification of infectious viruses, it is difficult to isolate single infectious virions from polyclonal viruses by this assay. Therefore, in addition to quantitative PCR, we used the plaque assay to quantify the infectious viruses and isolate the viruses as single clone. The plaque assay could quantify only the infectious viruses that were released from infected cells and could provide an infectious virus clone directly from a plaque because each plaque was derived from one infected cell [17]. One of the three monkeys used in this study developed AIDS. Our results showed that the viruses actively replicated not only in the lymphoid tissues but also brain of the monkey that developed AIDS and that the quasispecies of infectious viruses in the brain were different from those in the lymphoid tissues.

Materials and methods

Virus, monkeys, and inoculation

$SHIV_{KU-2}$ was provided by one of the authors (Dr. Opendra Narayan). It is a polyclonal chimeric virus derived by the in vivo passage of SHIV-4 [15, 24]. The virus stock used in this study was grown in rhesus macaque PBMC, and stored at −80 °C until use. Three adult rhesus macaques (*Macaca mulatta*) were used in this study. The monkeys were anesthetized by intramuscular injection of ketamine chloride and intravenously inoculated with 1×10^5 $TCID_{50}$ of the virus stock. Two monkeys, MM254 and MM255, were euthanized at 6 weeks post inoculation (p.i.) and one monkey, MM201, which developed an AIDS-like disease was euthanized at 26 weeks p.i. Throughout the experimental period, the monkeys were housed in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University.

Sample collection

Blood was periodically collected from all monkeys. PBMC and plasma were separated from heparinized blood by Percoll (Nacalai Tesque, Kyoto, Japan) density gradient centrifugation. Plasma was frozen at −80 °C until use. Brain and tissues from various lymphoid organs were obtained at the time of euthanasia. A brain tissue sample was obtained from cerebral cortex, and bone marrow was harvested from the femur. Parts of the samples were frozen directly at −80 °C until use for quantification of proviral DNA. Residual samples of brain, spleen, thymus, bone marrow, mesenteric lymph node and a mixture of axillary and mandibular lymph nodes were minced and filtered through a 40 μm nylon filter (Becton Dickinson, Franklin Lakes, New Jersey). The obtained cells were immediately used in the plaque assay.

Quantification of plasma viral RNA

The viral RNA loads in plasma were determined by quantitative RT-PCR [34]. Total RNAs were prepared from plasma with a QIAamp Viral RNA kit (QIAGEN, Hilden, Germany). RT-PCR was performed with a Taqman EZ RT-PCR kit (Perkin Elmer, Wellesley, MA) for the SIV gag region using the following primers: SIV2-696F (5′-GGA AAT TAC CCA GTA CAA CAA ATA GG-3′) and SIV2-784R (5′-TCT ATC AAT TTT ACC CAG GCA TTT A-3′). A labeled probe, SIV2-731T (5′-Fam-TGT CCA CCT GCC ATT AAG CCC G-Tamra-3′; Perkin Elmer), was used for detection of the PCR products. These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and analyzed using the manufacturer's software. For each run, a standard curve was generated from dilutions whose copy numbers were known, and the RNA in the plasma samples was quantified based on the standard curve.

Quantification of proviral DNA

The proviral DNA loads in tissues were determined by quantitative PCR. DNA samples were extracted directly from frozen tissues with a QIAGEN DNeasy Tissue kit (QIAGEN). PCR was performed with a Taqman PCR Reagent kit (Perkin Elmer) using the same primer set and probe which were used in RT-PCR. A standard curve was generated from a plasmid DNA sample containing the full genome of SHIV NM-3rN, which was quantified with a UV-spectrophotometer.

Plaque assay

The infectious viruses were quantified and isolated using the plaque assay [17]. An agarose gel bilayer containing RPMI 1640 medium was made in plastic culture dishes of 100 mm; the

lower layer consisted of 12 ml of 1.2% agarose (Agarose NA; Pharmacia, Uppsala, Sweden) and the upper layer consisted of 12 ml of 0.4% low-gelling-temperature agarose (SeaPlaque Agarose; FMC, Rockland, ME). The dishes were incubated at 37 °C in 5% CO_2 overnight. The following day, 2×10^6 cells of each sample and 8×10^6 cells of M8166 were suspended in 3 ml of a 0.4% low-gelling-temperature agarose solution containing the culture medium and the mixture was immediately overlaid on the agarose gel layer previously prepared. To detect infectious viruses in the brain, approximately 10 cm^3 of brain tissue was homogenized and then mixed with M8166 cells. The mixture was suspended in agarose solution and overlaid on the plate. To assay the plasma, 8×10^6 M8166 cells were incubated with 500 μl of plasma in 1 ml of medium for 1 h. The cells were suspended in agarose solution, and this mixture was overlaid on the plate. After the gel was hardened, the plates were covered with 12 ml of culture medium and incubated at 37 °C in 5% CO_2 for 10 days. The medium over the plates was replaced with fresh medium every day. After removal of the medium in the plates at 10 days, the plates were stained with 1.8 ml of 0.7% MTT for 2 h to count the number of the plaques. Each plaque represented an infectious virus clone. A portion of a plaque on the agarose gel was picked up with a pipette tip and suspended in 500 μl of medium. After centrifugation, the cell pellet was stored at −80 °C.

Sequence analysis

Total DNA was extracted from the cell pellet with a DNeasy Tissue Kit (QIAGEN). The 365 bp of the *env* V3 region was amplified by PCR with one primer pair: V3BgF1 (5′-CAG GCC AGT AGT ATC AAC TCA ACT GC-3′) and YT001 (5′-ACA ATT TCT GGG TCC CCT CTG AGG A-3′), in a mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each dNTPs, 0.2 μM of each primer and 2.5 units of AmpliTaq DNA polymerase. The PCR products were purified with QIAquick Spin (QIAGEN) and directly sequenced with the sense primer V3BgF1 by a Taq cycle sequencing system using the Dyedeoxy termination method and analyzed with an ABI373A automated DNA sequencer (Applied Biosystems). The nucleotide sequences of the analyzed clones were converted into amino acid sequences and aligned with $SHIV_{KU-2}$ [25] using the Clustal W program [37]. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide sequence database with accession numbers AF364413 to AF364476.

Results

Infection of rhesus macaques with $SHIV_{KU-2}$

Three rhesus macaques, MM201, MM254 and MM255, were intravenously inoculated with $SHIV_{KU-2}$. In all monkeys, viral RNAs in the plasma increased rapidly after infection and viremia reached peak levels (10^6 to 10^7 copies/ml) at 2 weeks post inoculation (p.i.) (Fig. 1). Concurrent with the increase in viremia, $CD4^+$ T cell counts declined to 5 to 40% of normal range after the inoculation (data not shown). MM254 and MM255 were then euthanized at 6 weeks p.i. In the remaining monkey, MM201, plasma viral RNA remained high (in the range of 10^5 to 10^6 copies/ml) and $CD4^+$ T cell counts remained low (less than 100 cells/μl) throughout the infection. Finally, MM201 developed an AIDS-like disease with neurological defects at the time of euthanasia, at 26 weeks p.i. Neurobehavioral and histopathological abnormalities with encephalitis were observed in this monkey (data not shown).

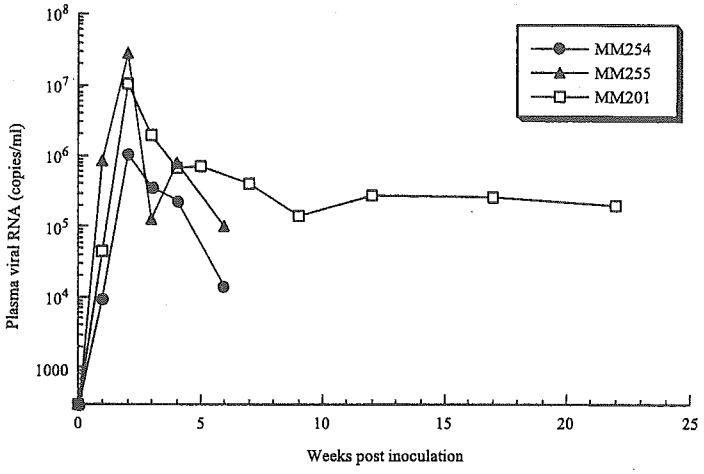

Fig. 1. Plasma viral RNA loads of three $SHIV_{KU-2}$-inoculated monkeys. MM255 and MM254 were euthanized at 6 weeks p.i. MM201, which developed an AIDS-like disease, was euthanized at 26 weeks p.i. The detection limit of this assay was 3×10^2 copies/ml

Quantification of proviral DNA

To examine the correlation between the amount of virus harbored in the various tissues of the individuals and the development of AIDS, the viral loads were quantified in the blood, brain and some lymphoid tissues of the monkeys at the early stage of infection (6 weeks p.i.) and at the AIDS stage (26 weeks p.i.). First, proviral DNA loads in the tissues were measured by quantitative PCR. In the early stage of infection, proviral DNA was detected in the PBMC, spleen, thymus and lymph nodes of the two monkeys (MM254 and MM255), ranging from 10^4 to 10^5 copies per microgram of total cellular DNA (Fig. 2). Proviral DNA was also detected in the brain tissues of both monkeys, although the copy numbers were lower than those in the other samples.

At the AIDS stage, the proviral DNA loads in all the tissue samples of the infected monkey (MM201) were higher (10 to 10^2 times) than those of the other two monkeys in the early stage of infection (Fig. 2). The proviral DNA load in the brain tissues was especially high, being 10^4 times higher than the loads in the other two monkeys. These results showed that the virus infected the brain tissues early after inoculation and increased at the late stage of infection.

Quantification of infectious viruses

Although the proviral DNA was present in each tissue of the infected monkeys, it was not clear which of the infected tissues released the infectious viruses that are considered to play a major role in virus spread in a body. Therefore, only the infectious viruses were quantified using the plaque assay in the various tissues of the infected monkeys. The infectious viruses were detected in PBMC, spleen,

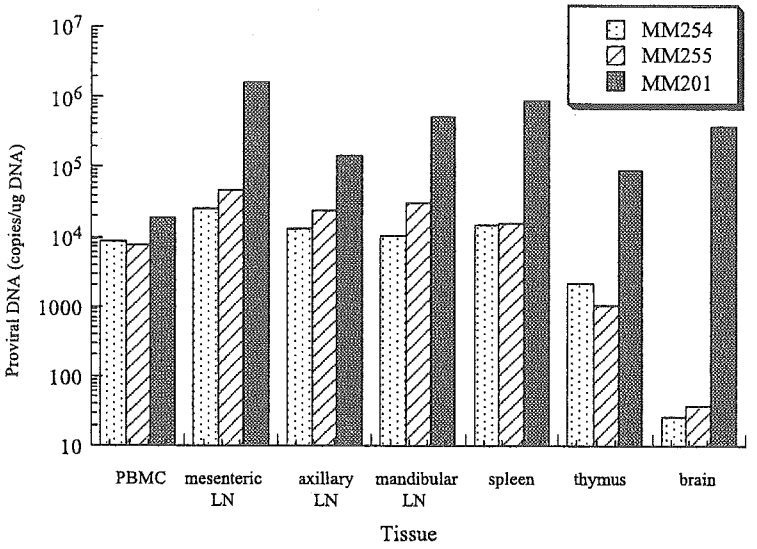

Fig. 2. Proviral DNA loads in various tissues of three $SHIV_{KU-2}$-inoculated monkeys. Viral loads were determined by quantitative PCR and are expressed as viral DNA copy numbers per microgram of total DNA extracted from the tissue homogenates. MM254 and MM255 were euthanized at 6 weeks p.i. MM201 was euthanized at 26 weeks p.i.

and mesenteric and axillary/mandibular lymph nodes of the two monkeys in the early stage of infection (Fig. 3). In MM254, the viral loads in PBMC, spleen, and lymph nodes were almost the same. In MM255, the viral loads in the spleen and axillary/mandibular lymph nodes were higher than those in PBMC and mesenteric lymph node. However, infectious virus was not detected in thymus, bone marrow and brain of both monkeys although proviral DNA was detected in these samples.

In the monkey that developed AIDS, MM201, infectious viruses were detected at various levels in all the tissues we examined, except for the thymus. The levels of infectious virus in the spleen, mesenteric and axillary/mandibular lymph nodes of MM201 were detected more than the level in PBMC, and the mesenteric lymph node of MM201 had an especially large number of infectious viruses (250 pfu per 10^6 cells). Moreover, in MM201, the infected cells that produced infectious viruses were present in the brain and bone marrow, but not in these same tissues in the other two monkeys early after infection. These results showed that the productive viral infection occurs in the brain tissues at the AIDS stage of infection, not early after infection.

Quasispecies analysis

To characterize the genotype of the viruses that replicated in each tissue of the infected monkeys and caused AIDS, the 270-bp nucleotide sequence encompassing HIV-1 *env* V3 region, which is an immunologically important domain, was analyzed in the infectious viruses isolated from each plaque. Similarly, the viral

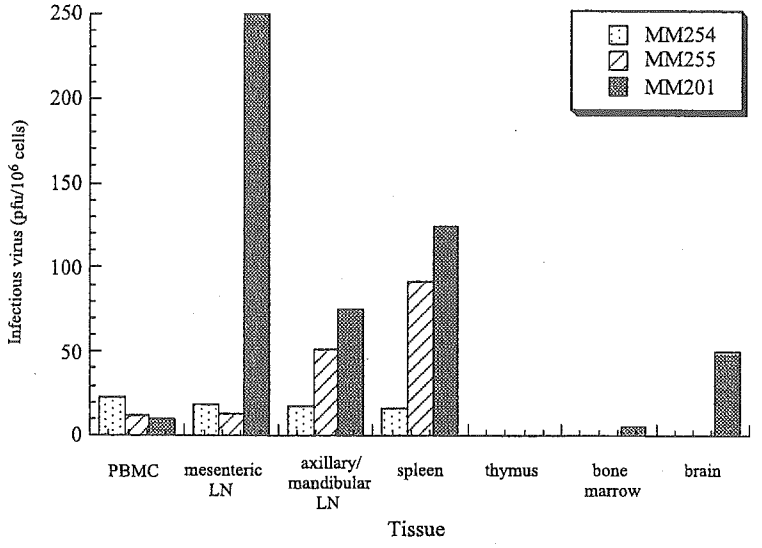

Fig. 3. Infectious virus loads in various tissues of three $SHIV_{KU-2}$-inoculated monkeys. Viral loads were determined by plaque assay and are expressed as the number of plaque forming units (pfu) per 10^6 cells or 10 cm^3 of brain tissues. MM254 and MM255 were euthanized at 6 weeks p.i. MM201 was euthanized at 26 weeks p.i.

genotypes in the virus stock were analyzed after isolation of the viral clones by plaque assay, resulting in a single genotype (Fig. 4).

Among the infectious viruses in different tissues of MM201, which developed AIDS, the nucleotide sequences encompassing the *env* V3 region varied greatly from the sequence of the inoculated virus. Moreover, three of the amino acid substitutions were commonly found in these sequences. One substitution [threonine (T) to lysine (K) or alanine (A)] was detected at a glycosylation site in the V3 region and the others [alanine (A) to threonine (T), phenylalanine (F) to tyrosine (Y)] were detected downstream of the V3 region, i.e., in the C3 region. Each viral genotype was named by three letters corresponding to these amino acid substitutions (Fig. 4): TAF, AAF, TAY, TTY, KAF, KAY, KTF, and KTY. In the PBMC, spleen, lymph nodes and bone marrow, all the viral genotypes, except for two clones, had both substitutions, i.e., the T-to-K substitution and the F-to-Y substitution. Moreover, the substitution from A to T was detected in 63% of all the genotypes in these tissue samples. Thus, at the AIDS stage of infection, the viral genotypes in most lymphoid tissues diverged from the virus stock. However, most of the viral genotypes in the brain were identical to that of the virus stock although two clones of the KAF and KAY types were detected. These results showed that the infectious viruses in the brain tissues were different from those in the lymphoid tissues, and that the infected cells in the brain tissues could produce both the original viruses and the diverged viruses at the AIDS stage of infection.

We next analyzed the nucleotide sequences of the infectious viruses from the other two monkeys at the early stage of infection, focusing on the viral genotypes

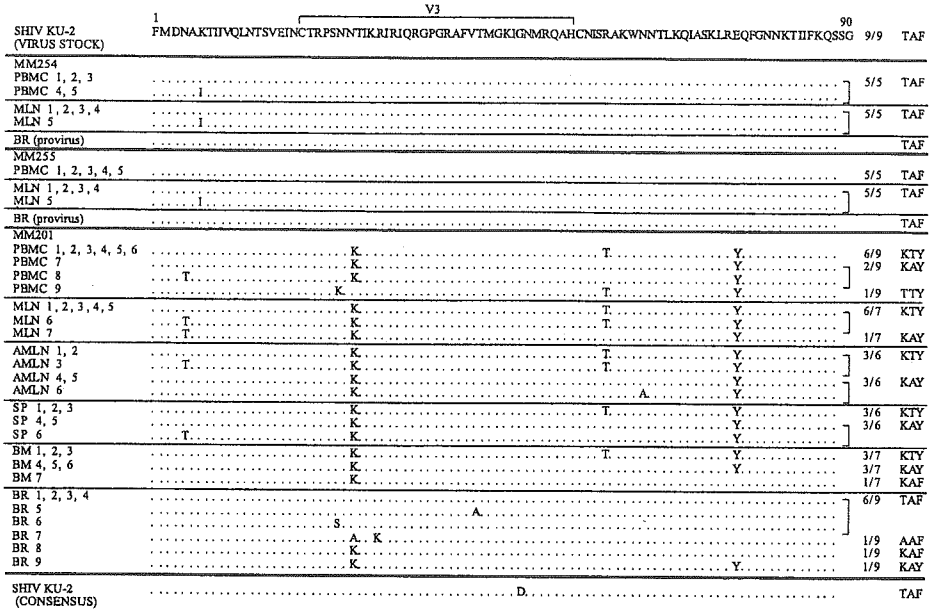

Fig. 4. Comparison of amino acid sequences of the HIV-1 *env* V3 region of infectious virus clones obtained from the $SHIV_{KU-2}$ virus stock and the $SHIV_{KU-2}$-infected monkeys by plaque assay and proviruses in the brains of MM254 and MM255. MM254 and MM255 were euthanized at 6 weeks p.i. MM201 was euthanized at 26 weeks p.i. Shown below the sequences is the consensus sequence of $SHIV_{KU-2}$ reported previously. A dot represents an amino acid identical to the sequence of the virus stock. Numbers at the right represent the total number of clones with a particular genotype for each tissue. The genotype of each sequence is represented by the residues at the three substitution sites. *MLN*, mesenteric lymph node; *AMLN*, axillary/mandibular lymph nodes; *SP*, spleen; *BM*, bone marrow; *BR*, brain

in PBMC and mesenteric LN which genetically diverged in the monkey at the AIDS stage of infection. Most of the viral genotypes in the PBMC and mesenteric lymph nodes of both monkeys were identical to that of the inoculated virus, although a single amino acid substitution upstream of the *env* V3 region was detected in some genotypes. Moreover, the sequences analysis of proviral DNA in the brain of both monkeys showed that the viral genotypes in the brain of the monkeys early after infection were also identical to that of the inoculated virus. These results supported that the viruses, which did not undergo genetic change, existed not only in the lymphoid tissues but also in the brains at early stage of infection.

Discussion

We quantified proviral DNA and infectious virus in various tissues of $SHIV_{KU-2}$-inoculated monkeys in the early and AIDS stages of infection. The proviral DNA and the infectious viruses were equally detected in PBMC of the infected monkeys

regardless of the infection period. However, the viral load in the lymphoid tissues was higher at the AIDS stage than at the early stage of infection. Especially, the mesenteric lymph nodes showed the greatest increases in the number of infectious viruses at the AIDS stage compared with the number of infectious viruses early after infection, although all three monkeys had almost the same plasma viral RNA loads. These results were confirmed by the quantification of both viral DNA and infectious virus. A previous study showed that mesenteric lymph nodes contain numerous SIV-infected cells at all stages of SIV-infection [12]. In the gastrointestinal tract, many lymphoid cells are constantly activated by antigenic stimuli, including food. Because HIV/SIV could replicate optimally in the activated T cells, the gastrointestinal tract might be a major site of viral replication at the time of development of AIDS.

Based on the sequences encompassing the *env* V3 region, the infectious viral populations in PBMC and mesenteric lymph nodes of the two monkeys at the early stage of infection hardly diverged from the inoculated virus. However, the sequences of infectious viruses in the monkey that developed AIDS revealed that the viral populations in PBMC and lymphoid tissues diverged from the inoculated virus and had three common amino acid substitutions in the predicted sequences of the infectious viruses. One substitution [threonine (T) to lysine (K)] was detected at the glycosylation site in the V3 region. A T-to-K substitution at this site could add a positive charge and abolish a potential N-linked glycosylation site. This substitution in HIV and SIV was reported to be correlated with a switch of the viral phenotype and a change in the interaction between the virus and the host immunity [9, 29]. Moreover, this site was also reported to be an epitope of cytotoxic T lymphocytes (CTLs) [30]. The other substitutions [alanine (A) to threonine (T) and phenylalanine (F) to tyrosine (Y)] were detected downstream of the V3 region, in the C3 region. Because this region was included in the outer domains of the envelope [22], it is possible that amino acids located on this region represent targets for CTLs or antibodies. Therefore, these infectious viruses with the amino acid substitutions might escape from the immune response in the infected monkeys and cause the development of AIDS.

The brain was reported as one of the tissues which the virus latently infected [10, 18, 20, 32, 35]. However, little is known about when the virus crosses the blood-brain barrier and infects the cell in the brain although various analyses about neuropathogenesis were performed in patients with HIV dementia. In this study, proviral DNA, but not infectious virus, was also detected in the brain of the monkeys in the early stage of infection. In contrast, not only proviral DNA but also infectious virus was detected in the brain of the monkey that developed AIDS with encephalitis. These results suggested that the brains of the infected monkeys were exposed to the virus during the acute infection but that the virus might hardly replicate in the brain. In SIV-infected macaques, both proviral DNA and viral RNA were detected in the brain during the acute infection [23, 26]. However, the detection of viral RNA was transient, whereas proviral DNA latently existed in the brain throughout infection [6]. Following persistent infection in the brain, viral RNA and viral protein, in addition to proviral DNA, were reproduced in the brain

at the late stage of infection [3, 7, 40]. In our study, with the progression of AIDS disease, the latent infection might be broken and the production of the infectious virus increased in the brain. Establishment of a productive infection in the brain might result in the neurological lesions in AIDS.

Previous studies showed genetic differences between the viral sequences derived from the blood and brain of HIV-1 infected patients [2, 19]. In our study, based on the sequence encompassing the *env* V3 region, the infectious viral population in the brain of the monkey that developed AIDS with encephalitis was similar to the inoculated virus, while that in the lymphoid tissues genetically diverged from the inoculated virus. The infectious viruses produced in the brain at the AIDS stage of infection resulted from the viruses which entered the brain early after infection and had persisted in the brain without genetic divergence. For individuals at the AIDS stage, immunological changes might trigger active viral replication in the brain and then result in neurological defects. Further studies of the evolution of the virus in different tissues may eventually lead to better methods for suppressing replication.

Acknowledgments

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Human Astrovirus, Norovirus (GI, GII), and Sapovirus Infections in Pakistani Children With Diarrhea

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Fecal specimens from 517 infants and young children admitted to the Civil Karachi Hospital, Dow Medical College, Karachi city, Pakistan with acute gastroenteritis from 1990 to 1994 were collected and screened by RT-PCR for human astrovirus (AstV), norovirus (NV), and sapovirus (SV). The specific epidemiological data for illness caused by these viruses in Pakistan are not available. AstV, NV, and SV were detected in 58, 51, and 17 of 517 fecal specimens, and this represented 11.2, 9.9, and 3.2%, respectively. An outbreak of gastroenteritis attributable to AstV serotype 1 was identified during September and October 1990. Moreover, one specimen with a triple mixed infection between AstV (serotypes 1 and 3) and NV GII was found. NV and SV were subjected to molecular analysis by sequencing. One of the sequenced specimens positive for SV turned out to be similar to a strain tentatively called a genogroup IV. The result underscores the importance of these viruses in association with acute gastroenteritis in Karachi city, Pakistan. ***J. Med. Virol. 73:256–261, 2004.***

KEY WORDS: multiplex PCR; serotype; genotype; enteropathogen

INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to gastroenteritis is greater in developing than in the developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths. Apart from group A rotavirus as the most common cause of gastroenteritis, norovirus (NV), and sapovirus (SV) are considered to be significant global enteropathogens. These viruses also are associated with sporadic outbreaks of gastroenteritis in such settings as kindergartens, schools, and nursing homes for the elderly and among military recruits [Carter and Willcocks, 1996; Bon et al., 1999].

Astrovirus (AstV), so called to describe the distinctive five-or six-pointed star visible on some particles when viewed under the electron microscope (EM), is a small, 28-nm-diameter, nonenveloped, single capsid layered viruses with a positive-sense single-stranded RNA genome. This virus has three open reading frames (ORFs)—ORF1a, ORF1b, and ORF2. Moreover, ORF1a and ORF1b at the 5, end of the genome encode the viral protease and polymerase, respectively, whereas ORF2 at the 3, end of the genome encodes the capsid protein precursor [Schnagl et al., 2002]. AstV is classified currently into eight serotypes [Sakamoto et al., 2000].

NV and SV contain a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The NV genome contains three ORFs (ORF1, 2, and 3); ORF 2 encodes the capsid proteins [Glass et al., 2000]. In the two SV ORFs (ORF 1 and 2), however, it is ORF 1 that encodes the non-structural as well as the capsid proteins. Based on the sequence analysis of the capsid gene, both human NV and human SV are divided into two genogroups (I, II) [Schuffenecker et al., 2001].

The objectives of this study were: to describe the prevalence of AstV, NV (GI, GII), and SV in fecal

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