

FIGURE 1. Expression of LacZ gene in the rat liver after catheter-based selective injection of large-volume naked DNA solution. Liver with X-gal staining after selective injection of 125 μg naked LacZ gene diluted in 3.0%, 2.5%, 2.0%, and 1.5% of body weight phosphate-buffered saline (PBS) (A). Liver after 2.5% volume of rat body weight PBS and 125 μg naked LacZ are stained with X-gal and eosin (B). Upper row magnification $\times 40$; lower row magnification $\times 100$.

TABLE 2. Graft survival after heterotopic heart transplantation to pCAG CTLA4Ig-transfected recipient using the CB-RILV method

Group	Gene therapy		N	Graft survival (d) (mean \pm SD)	Serum CTLA4Ig level ($\mu\text{g/mL}$) ^a		
	Method	CTLA4Ig (μg)			Day 0	Day 2	Day 5
1	—	—	6	5, 6, 6, 6, 6, 7 (6.0 \pm 0.6)	NT	NT	NT
2	CB-RILV	125	6	5, 5, 6, 39, 39, 42 (22.7 \pm 19.0)	22.7 \pm 18.4	14.5 \pm 13.1	9.9 \pm 11.3
3	CB-RILV	250	6	5, 5, 6, 10, 26, 32 (14.0 \pm 11.9)	19.7 \pm 15.3	14.5 \pm 8.6	7.3 \pm 5.6

^a Data are expressed as the mean \pm SD.

NT, not tested; SD, standard deviation; Ig, immunoglobulin; CTLA, cytotoxic T-lymphocyte antigen; CB-RILV, catheter-based rapid injection of large volume solution with naked DNA.

control group. There was no significant difference between groups 2 and 3. No correlation was found between serum CTLA4Ig levels on days 0, 2, and 5 and graft survival times. There was no significant difference between groups 2 and 3 in serum CTLA4Ig levels on days 0, 2, and 5 after transplantation.

Application of Rat Auxiliary Liver Transplantation and Orthotopic Liver Transplantation

We first evaluated the timing of gene transfer to the liver graft using ALT, which is associated with lower mortality than that of OLT. As shown in Table 3, seven of nine recip-

TABLE 3. Recipient survival after alanine aminotransferase using gene-transfected graft liver by the CB-RILV method

Timing of gene therapy	Donor	Recipient	No. rats	Recipient survival (d)
2 d	Lewis	Lewis	3	>14, ^a >20, ^a >23 ^a
0 d	Lewis	Lewis	9	0, 0, 0, 0, 1, 2, 4, >21, ^a >23 ^a
None	Lewis	Lewis	5	>30, >30, >30, >30, >30

^a Recipients were killed for histologic study.

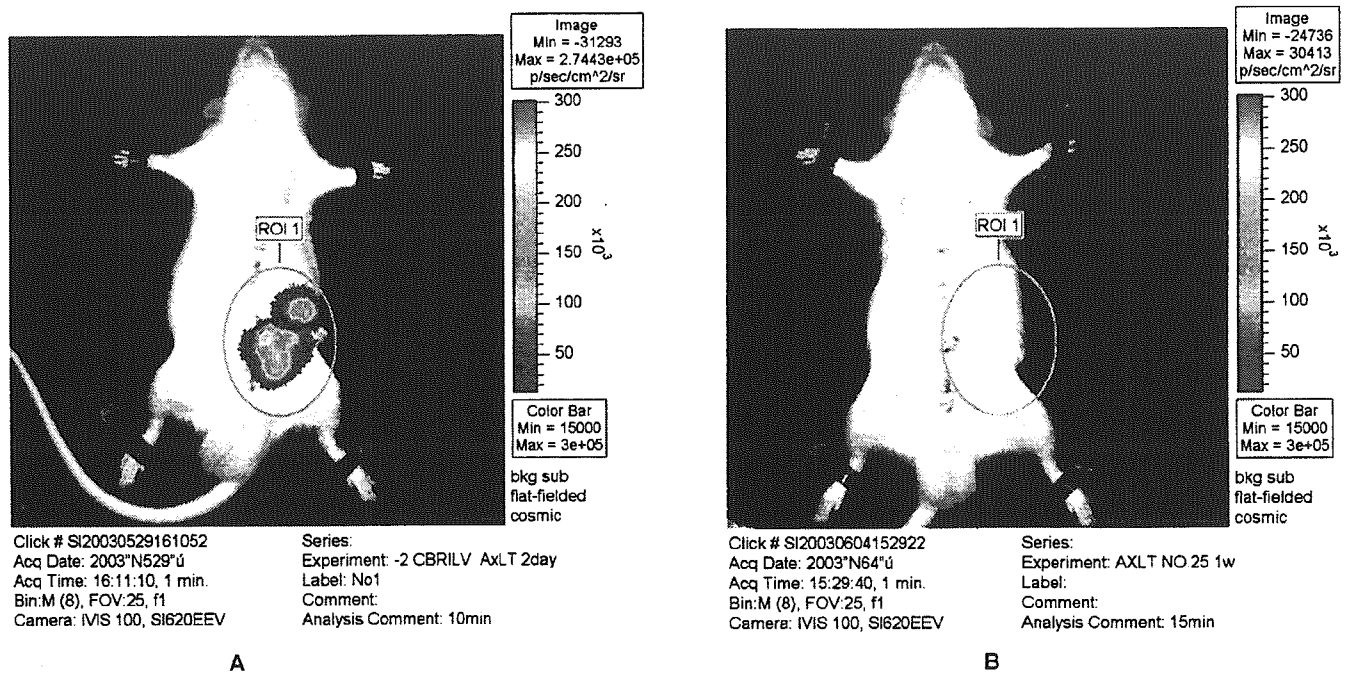


FIGURE 2. Expression of the luciferase gene in the graft liver after auxiliary liver transplantation (ALT). Two days before transplantation, 2.5% volume of donor body weight PBS and 125 μ g of pGL3 were injected using a catheter-based selective injection method. Efficient expression of pGL3 was observed 2 days after transplantation (A) but decreased to an undetectable level 1 week after transplantation (B).

ients died of graft congestion or venous thrombosis 0 to 4 days after ALT when gene therapy was delivered to the graft on the day of transplantation. All recipients survived when gene therapy to the graft was performed 2 days preoperatively. The expression of pGL3 after ALT is shown in Figure 2. Gene expression in the graft liver was detected 2 days after ALT but was undetectable 10 days after ALT.

On the basis of these results, we decided to use liver grafts that underwent gene transfection 2 days before OLT. The pGL3 or pCAG-CTLA4Ig expression in the graft liver after OLT is shown in Figure 3. One day after OLT, luciferase expression was readily detected. Recipient serum CTLA4Ig increased 2 days after transplantation and decreased to less than 5 μ g/mL 5 days after transplantation ($n=4$).

DISCUSSION

A combination of gene therapy for the liver and transplantation is a strategy for various situations including induction of transplant tolerance, treatment of recipients with metabolic disorders, and DNA vaccination for treatment of fulminant hepatitis (23). Moreover, the nonviral gene transfer method is beneficial for recipients who receive immunosuppressive agents, in whom the clinical applications of virus

vectors may be limited because of cytotoxicity and immune responses (8, 9). Delivery of the naked DNA using an intravascular route results in effective gene transfer to hepatocytes because the vascular system accesses every cell (15). We investigated this method and the manner in which the combination therapy of gene transfer and transplantation was applied.

We found that 125 μ g of naked DNA diluted with PBS in a volume of 6.25% of body weight is the best protocol in rats for efficient gene expression and minimal damage to the host. There are three important factors for systemic administration of naked DNA: retrograde injection into the liver, a large volume of DNA solution, and rapid injection (10, 11). When naked DNA is injected into the vein, serum DNAase rapidly degrades the plasmid cDNA within 5 min, and it is taken up by hepatic nonparenchymal cells (24, 25). Rapid injection of a large volume of solution may help overcome the effects of degradation by DNAase and uptake by hepatic nonparenchymal cells. Moreover, rapid injection of a large volume of solution contributes to an increase in the hepatic venous pressure. Song et al. (16) reported that rapid injection of a large volume of DNA solution exceeds the cardiac output, resulting in accumulation of DNA solution in the IVC, and

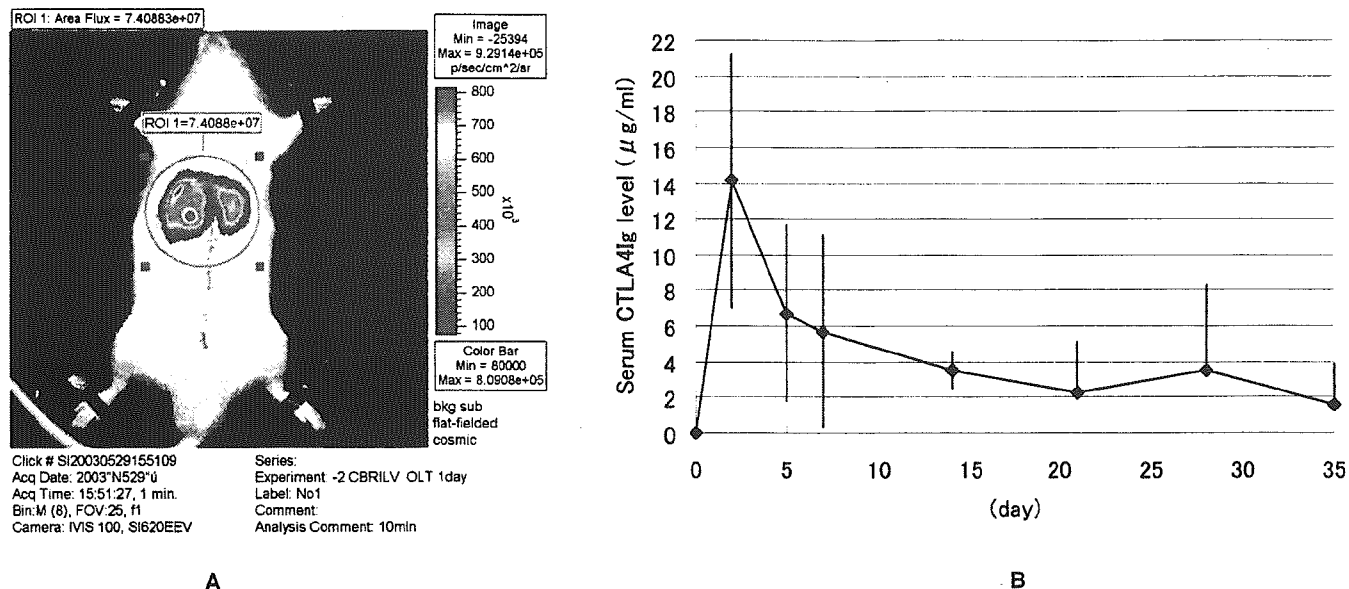


FIGURE 3. Expression of the luciferase or cytotoxic T-lymphocyte antigen 4-immunoglobulin (*CTLA4Ig*) gene in the graft liver after orthotopic liver transplantation (OLT). Each gene was transfected using the same method as in Figure 2. Efficient expression of pGL3 was observed 1 day after OLT (A). Transient production of CTLA4Ig protein was detected in recipient peripheral blood samples (B) (n=4).

high pressure in the hepatic vein contributes to efficient gene transfer. However, our data showed that transient increase of CVP, which is considered to correlate with the hepatic venous pressure, and transient cardiac failure (indicated by decreased systemic blood pressure, elevated CVP, and transient bradycardia) occurs during and immediately after injection. A direct increase in hepatic venous pressure might, therefore, contribute to efficient DNA transfection in our model.

Potential contamination of plasmid DNA by lipopolysaccharides is an important consideration because it may alter immune responses in transplant recipients (26). Although we performed CsCl ultracentrifugation during plasmid purification, the level of lipopolysaccharide contamination might be still substantial. However, 125 µg DNA (2.5% volume of body weight) showed little liver damage, and AST and alanine aminotransferase increase was minimal or negligible compared with that of normal rats. In addition, the same hydrodynamic procedure was also applied to the muscle in our previous study (17) without complications.

Rats injected rapidly with a solution greater than 7.5% die from pulmonary bleeding (unpublished data) as a result of damage to the heart and lungs. To reduce such damage, we focused on perfecting the technique of selective injection of the liver. Eastman et al. (27) recently demonstrated the catheter-based procedure for naked DNA transfection to the rabbit liver. With catheter-based injection, the total injection volume can be decreased, which selectively limits the liver damage. We established the catheter-based naked DNA transfection method for rats without mortality and succeeded in decreasing the injection volume to 2.5% of body weight.

We previously observed long-lasting gene expression in rat limb transplantation after rapid injection of naked DNA (17). However, gene expression after liver transplantation was transient. We also observed early down-regulation in a pre-

vious study in which the gene gun was used to deliver naked HBs gene to the graft liver (23). This phenomenon might be specific to the liver. Damage resulting from the injection of solution might induce liver regeneration. However, blocking the costimulatory pathway in the early phase after transplantation might be important for tolerance induction or prolonged graft survival (28–30). As shown in the present study, prolongation of allograft survival is achieved in recipients transfected with pCAG-CTLA4Ig. However, only 50% of grafts showed prolonged survival, and neither graft survival nor the recipient serum CTLA4Ig level related to the quantity of injected pDNA

As shown in this study, transfected cDNA using the CBRILV method was efficiently expressed in recipients after ALT and OLT. Because of the damage to the graft liver caused by the injection, many recipients died of graft congestion or a thrombus when gene therapy was performed on the day of transplantation, and 2 days were needed to allow the graft to recover from liver damage after injection. However, targeting gene therapy to a selected liver lobe using a catheter offers a clinically applicable approach. Immunomodification by the CTLA4Ig gene and DNA vaccine by antiviral genes of the graft liver are potential tools.

CONCLUSION

We applied a novel gene transduction treatment to the rat liver using a nonviral method. This is the first study to report successful gene expression after intravascular delivery of naked DNA to the donor liver before liver transplantation.

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Excision of pseudotumour in a patient with haemophilia A and inhibitor managed with recombinant factor VIIa

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Summary. We describe a patient with haemophilia A and factor VIII inhibitor who underwent surgical excision of a large pseudotumour in the left femoral region. Haemostasis was successfully maintained with bolus doses of recombinant factor VIIa at 2-h intervals and anti-fibrinolytic therapy, and the pseudotumour was removed. Subsequently, the dose interval was gradually prolonged to 8 h for a total

of 18 days. Although a spontaneous haemorrhage was observed on postoperative day 8, haemostasis was achieved by reducing the dosage interval. No adverse event occurred during the course of treatment.

Keywords: haemophilia A, inhibitor, pseudotumour, recombinant factor VIIa, surgery

Introduction

For many years prothrombin complex concentrates (PCC) and activated PCC have been used as haemostatic agents for haemophilia patients with inhibitors. Surgical procedures in these inhibitor patients were challenging because no single measure provided safe and reliable haemostasis. As recombinant factor VIIa (rFVIIa) product became available, a number of orthopaedic procedures including arthroscopic and surgical synovectomy and total joint arthroplasty in inhibitor patients managed with rFVIIa have been reported [1–6]. However, surgical case reports of pseudotumour resection are still limited [4,7,8]. We report the surgical removal of a pseudotumour in the left thigh of a patient with haemophilia A and inhibitor managed with rFVIIa.

Case report

On 16 November 2000, a 52-year-old Japanese man with haemophilia A and factor VIII inhibitor was

admitted to our hospital for removal of an infected haematoma in the left femoral region. He had had recurrent intra-articular haemorrhage since childhood. It was unclear when he developed the inhibitor. In September 1999, swelling was noted in the left femoral region but was not treated. In May 2000, the intramuscular haematoma in the femoral region enlarged, and skin ulcers developed with local infection. The man was admitted to a local hospital where a 1 BU mL⁻¹ of FVIII inhibitor was noted. After administration of a large dose of FVIII concentrate, the inhibitor titre increased to 108 BU mL⁻¹. He was referred to another hospital where he was treated with rFVIIa, resulting in some regression of the haematoma.

On admission, the patient's FVIII inhibitor titre was 17.5 BU mL⁻¹ with an FVIII activity of 0.017 BU mL⁻¹. He had a pseudotumour (20 × 10 cm) in the left femoral region with two skin ulcers of 2 cm in diameter in the posteromedial area. *Serratia marcescens* was isolated from exudation cultured from the skin ulcers. The patient showed muscle weakness in the left lower extremity due to sciatic neuroparalysis and had sensory disturbance in the leg. The left knee joint showed contracture in extension with no mobility. A preoperative rFVIIa pharmacokinetic study was performed (Fig. 1). Plasma factor VII activity increased up to 71 U mL⁻¹ 10 min after the dose of rFVIIa

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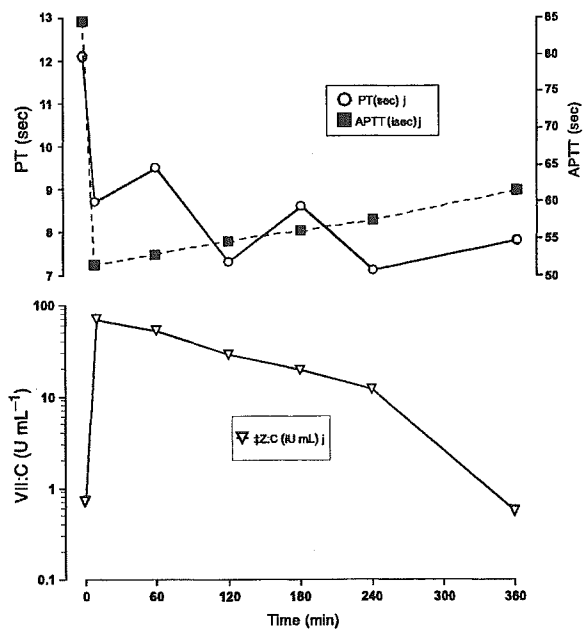


Fig. 1. Recombinant factor VIIa (rFVIIa) administration test. After bolus administration of 6 mg ($110 \mu\text{g kg}^{-1}$) rFVIIa, prothrombin time (PT), activated thromboplastin time (APTT), and factor VII activity by one stage assay were measured over a 360-min period.

($110 \mu\text{g kg}^{-1}$) and decreased with a half-life of 1.67 h. Although activated partial thromboplastin time (APTT) shortened from 84.5 to 51.3 s and gradually returned to 61.5 s over a period of 360 min, shortening of prothrombin time (PT) did not correspond with the level of plasma FVII activity.

Surgery was performed on 29 November 2000. The first dose of rFVIIa ($110 \mu\text{g mL}^{-1}$) was given immediately before intratracheal intubation. The dose was then repeated every 2 h. Tranexamic acid was also administered intravenously (13.6 mg kg^{-1} every 6 h) to inhibit fibrinolysis. Surgery began 1.5 h after administration of the first dose of rFVIIa. Haemostasis was achieved, although profuse haemorrhage was noted from the skin incision after the second dose of rFVIIa. During the operation, the sciatic nerve was not exposed in the surgical field. Although the pseudotumour was eventually removed, it was adherent to the soft tissue and too massive to be evacuated *en bloc*. An indwelling suction tube was placed in the incision site where a compression bandage was applied. The operating time was 3 h and 14 min with intraoperative blood loss of 1608 mL. The interval of rFVIIa administration was tapered from postoperative day 3. Concomitant tranexamic acid was switched to oral administration from day 3 (Table 1). On postoper-

Table 1. Administration of rFVIIa and tranexamic acid.

Days after operation	Interval (h) of rFVIIa administration (110 g/kg)	Dosage of tranexamic acid at 6-h intervals
1–2	2	750 mg i.v.
3–4	3	750 mg p.o.
5–6	4	
7	5	
8	6	
9–10	4.5	
11–12	5	
13–15	6	500 mg p.o.
16–17	7	
18	8	
19	4*	

*Dosage interval was decreased for prophylactic haemostatic coverage during stitch removal.

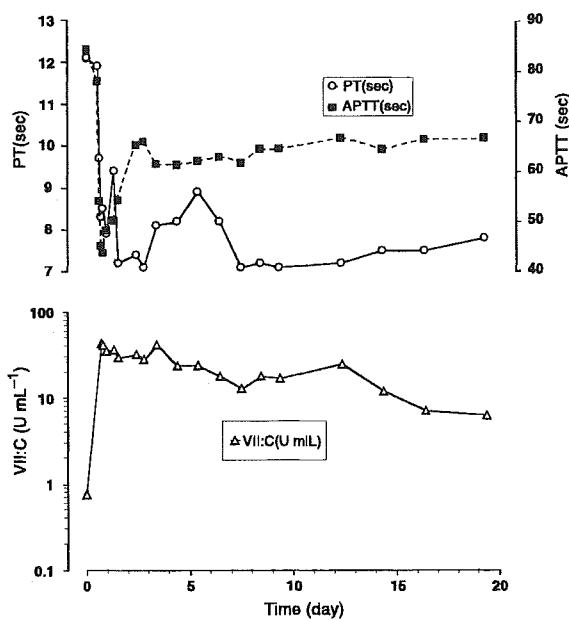


Fig. 2. Prothrombin time (PT), activated thromboplastin time (APTT), and factor VII activity (VII:C) during and after surgery. All values represent trough levels.

ative day 8, the patient complained of a sensation of tension and fullness in the left femoral region, where a haemorrhage was revealed by a computed tomography (CT) scan. Accordingly, the interval of administration of rFVIIa was shortened from 8 to 4.5 h, resulting in the improvement of the abnormal sensation with local haemostasis. On postoperative day 18, his body temperature rose to 38°C . Despite an antibiotic (cefpirome) that had been given for 5 days postoperatively, *S. marcescens* was isolated again from the surgical wound. Thereafter, a combination

of three antibiotics (cefpirome, levofloxacin and panipenem/betamipron) was given, and the infection subsided. On day 19, the suture stitches were removed, and rFVIIa administration was completed. PT and APTT remained short until the dose of rFVIIa was discontinued. Plasma FVII activity decreased as the dosage interval was prolonged (Fig. 2). There were no haematological findings or systemic activation of coagulation to raise suspicion of disseminated intravascular coagulation (DIC) during the course of treatment. After the infection was eradicated, the patient was discharged ambulatory with the aid of an apparatus and a cane.

X-ray findings

Plain X-ray examination revealed that the pseudotumour did not erode the femoral bone. CT scanning disclosed a uniform, low-density haematoma with no infiltration into the bone.

Discussion

False pseudotumours are chronic collections of blood in the muscles that do not affect bone structure. Once established, false pseudotumours tend to spread to bone or soft tissue lesions that lead to further neurovascular complications caused by vessel and nerve compression (true pseudotumours). Several treatment options are available for pseudotumours: embolization, irradiation, percutaneous aspiration, surgical evacuation and surgical excision of the pseudotumour [9–12]. Amputation is an extreme treatment option which is only rarely chosen even if the pseudotumour affects the bone and disables the extremity [13]. In the present case, although the pseudotumour had not affected the femoral bone, we hesitated to continue conservative therapy due to the complications of sacral nerve palsy and infected skin ulcers. As the patient wished to preserve the affected extremity, we chose excision of the pseudotumour with amputation of the affected extremity reserved as a salvage method.

Haemostasis was successfully maintained with bolus doses of rFVIIa at 2-h intervals, and the pseudotumour was removed. Subsequently the length of time between doses was prolonged over a 19-day period. A spontaneous local haemorrhage occurred on day 8, and the infection with *S. marcescens* recurred on day 18. We assume that a small amount of infectious tissue remained with inflammation because the pseudotumour was not evacuated *en bloc*, resulting in the postoperative haemorrhage in the left femoral region.

In recent years, elective surgery on haemophilia patients with inhibitor and use of rFVIIa has increased. Ingerslev *et al.* reported that seven of seven major surgical procedures in inhibitor patients were successfully managed with rFVIIa [14]. Lusher *et al.* summarized 103 surgical cases managed with rFVIIa in which haemostasis was evaluated as either excellent or effective in 17 of 21 major surgeries [15]. These experiences suggested that major surgery of haemophilic inhibitor patients can be performed quite safely by management with rFVIIa. A large number of inhibitor patients suffer from chronic arthrosis, and some patients incidentally develop pseudotumours, as in the present case. Potential advantages of rFVIIa in patients who need orthopaedic surgical procedures include its viral-free recombinant nature and the fact that fewer adverse events such as thrombosis or DIC have been reported [16]. There are, however, a few reports of patients who developed DIC during treatment with rFVIIa. In one case, a 21-year-old haemophilic patient with inhibitor developed an infectious abscess in the left thigh and underwent surgical management with coverage by rFVIIa. He had low-grade consumption coagulopathy before surgery. During the surgical procedure, he developed fatal DIC. *Salmonella mbandaka* was cultured from the wound [17]. In a second case, a 47-year-old inhibitor patient with a massive pseudotumour in the left abdomen and pelvis was infected with gas-forming *Clostridia* in the haematoma. Excision of the pseudotumour was performed with haemostasis management by rFVIIa. During the days following surgery, he developed septic shock and DIC, which subsequently subsided [4]. In a third case, a 39-year-old inhibitor patient underwent removal of osteosynthesis material. Uncontrolled bleeding occurred during the procedure with DIC, which subsided thereafter [18]. It appears that local infection before a surgical procedure could have been a major cause of DIC development in the first two of these three cases. Although our patient's skin ulcers were infected by *S. marcescens*, no systemic activation of coagulation was observed during the course of rFVIIa treatment.

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◆原 著◆

日本人血友病B患者に認められた18種類の遺伝子変異

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血友病BはX連鎖性劣性遺伝形式を呈し、血液凝固第IX因子の質的・量的異常によって引き起こされる出血性疾患である。我々は20家系の日本人血友病B患者よりDNAを抽出し、第IX因子遺伝子(F9)解析を行った。PCRにより全エクソンおよびイントロン境界領域を増幅し、ダイレクトシーケンシングにより塩基配列を決定した。20名から18種類の点変異(ミスセンス変異11種類、ナンセンス変異4種類、スプライスサイト変異3種類)を検出した。挿入や欠失変異は認められなかった。エクソン2のAla28→Pro, エクソン4のGln50→Lys, エクソン8のLeu300→Pro, イントロン7のドナースプライス部位でのa→c変異の4例は未報告変異であった。F9の国際的な変異データベースには、現在までに896種類の遺伝子異常が登録されている。本研究から血友病Bの病因遺伝子異常はさらに多様性を有することが示唆された。

Key words: hemophilia B, factor IX gene, nucleotide sequence, point mutation, genotype

緒 言

血友病Bは血液凝固第IX因子(FIX)の質的・量的異常であり、関節出血や筋肉内出血などの反復症状を特徴とする。本症はX連鎖性劣性遺伝形式を呈し、血友病Aの約1/5の発症頻度¹⁾、本邦では2002年に842人の血友病B患者が報告がされている²⁾。血漿FIX活性値(FIX:C)の定量により、病型の表現型は、1%未満を重症型、1~5%を中等症、5%以上を軽症に分類する。また血友病Aなどの他の先天性疾患同様に、血友病Bでは活性値と抗原量に乖離を認める、いわゆる分子異常に基づく病型がある。すなわち、FIX抗原(FIX:Ag)の量的欠損であるCross-Reacting Material (CRM)-negative, FIX:Agが低値を示すCRM-reduced, FIX:Agはほぼ正常に存在するが比活性が低い

CRM-positiveに分類される³⁾。

第IX因子遺伝子(F9)はX染色体長腕上の末端側Xq26-27の間に存在し全長約34kbで、8つのエクソンと7つのイントロンより構成される⁴⁾。約2.8kbのmRNA⁵⁾から合成されるFIXは、461のアミノ酸残基のプレプロ前駆体として主に肝臓で合成され、28残基のシグナルペプチドが切断されてプロFIXとなる。さらにプロセッシング酵素により18残基のプロ領域が切断を受け、415残基からなる分子量約55,000の成熟タンパク質として血中に分泌される。一本鎖のFIXは活性型第XI因子や活性型第VII因子・組織因子複合体によって、最初にArg145-Ala146が、続いてArg180-Val181が加水分解され、35残基の活性化ペプチドを遊離し活性型FIX(FIXa)となる。

FIXは、NH₂基末端側から12個のγ-カル

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ボキシグルタミン酸 (Gla) を有する Gla ドメイン, 次に2つの EGF 様ドメイン (EGF-1, EGF-2), 活性化時に遊離する活性化ペプチド, そして COOH 基末端領域のカトリティックドメインから構成されている。また, FIX の遺伝子およびタンパク質構造は, ビタミン K 依存性凝固因子として, プロトロンビン, 第 VII 因子, 第 X 因子, プロテイン C や S などと高い相同性を有している⁶⁾。

1982 年に cDNA が単離され⁵⁾ 1985 年に F9 の全塩基配列⁴⁾ が決定されて以来, 血友病 B における遺伝子解析は, Single Strand Conformational Polymorphism や Denaturing Gradient Gel Electrophoresis などの変異検出スクリーニング法や Polymerase chain reaction (PCR)-サイクルシーケンスによる塩基配列決定法などの生化学的手法の発展と相まって, F9 の遺伝子異常に関する多くの知見が蓄積されてきた。

血友病 B の治療は, 従来から血液凝固因子製剤補充療法が有効に行なわれている。米国では, 約 10 年前から血友病に対する遺伝子治療の研究が本格的に開始され, 2003 年骨格筋を標的とした第 1 相臨床研究でアデノ随伴ウイルスベクターによる血友病 B 遺伝子治療の安全性が明らかになった⁷⁾。新しい治療法の開発に伴い, 遺伝子異常を含む患者個々の症例の情報が重要になる。そこで今回我々は, 本邦における血友病 B 患者の F9 遺伝子解析に着手した。本研究では 20 家系の日本人血友病 B 患者の遺伝子異常を検出することを目的とし, それらの遺伝子型と血漿レベルの表現型との関連について検討した。

対 象

対象は東京医科大学病院臨床検査医学科通院中の血友病 B 患者 20 人で, 遺伝子解析にあたりすべての患者及び対照者よりインフォームドコンセントを得た。年齢性別は 0 歳から 64 歳

の男性であった。

方 法

① FIX:C および FIX:Ag の測定

3.2% クエン酸ナトリウム加血漿を用いた。FIX:C は自動血液凝固能測定装置 (ACL300R) を用い, APTT 試薬 (IL-test™; Instrumentation Laboratory) と FIX 欠乏血漿 (George King) を用いて凝固一段法⁸⁾ で測定した。FIX:Ag はポリクロナール抗体を用いたサンドイッチ EIA 法 (ASSERACROM® FIX:Ag (Roche Diagnostica Stago)) を使用して測定した。FIX:C および FIX:Ag の測定検体は, 7 日間以内に血液製剤輸注歴が無いものを採取した。

② ゲノム DNA の調製

ゲノム DNA は, 患者末梢白血球より得た核分画を SDS および Proteinase K で処理した後, フェノール・クロロホルム法で抽出した。

③ PCR 法

F9 の 8 つのエクソンおよびそのイントロン境界領域は, 設計した 8 対のプライマー (表 1) を用い PCR で増幅した。24bp の大きさのエクソン 3 は, エクソン 2 から 188bp 下流に近接しているため, これら 2 つのエクソンは 1 つの PCR 産物としてまとめて増幅した。また, エクソン 8 は 548bp の大きさがあり 2 分割して PCR で増幅した。PCR は耐熱性 DNA ポリメラーゼとして TaKaRa *Taq*™ (Takara Shuzo) または TaKaRa LA *Taq*™ (Takara Shuzo) を用いて, 変性; 96°C, 30 秒, アニーリング; 57~62°C, 30 秒, 伸長; 72°C, 40~60 秒を 40 サイクルで増幅した。

④ ダイレクトシーケンス法

それぞれの PCR 産物は 3% NuSieve® 3:1 アガロース (Cambrex) ゲル電気泳動を行い, 目的の大きさのバンドを切り出し, QIAquick® Gel Extraction kit (Qiagen) を用いて精製した。精製物をテンプレートとし, Thermo Sequenase® core sequencing kit (Amersham) を用いたジデ

表 1 Location and sequence of the oligonucleotide primers used

Exon		Oligonucleotide sequence	Nucleotide number	
Exon 1	FWD	5'-(M13F)CACAGTGGCAGAAGCCCACG-3'	-177	-158
	REV	5'-(M13R)TTACCAACCTGCGTGCTGGC-3'	240	258
Exon 2-3	FWD	5'-(M13F)CAAAGTGTGAAGTTAACCGC-3'	6119	6137
	REV	5'-(M13R)CCTGGGGTGAACAATGTTC-3'	6924	6943
Exon 4	FWD	5'-(M13F)TCTACAGGGGAGGACCGGGC-3'	10324	10343
	REV	5'-(M13R)CATTTTCCAGTTTCAACTTG-3'	10555	10574
Exon 5	FWD	5'-(M13F)CATGTACTTTTGTAGAAATGC3'	17608	17627
	REV	5'-(M13R)ATGTAGTTTGTAAAATGG3'	17848	17867
Exon 6	FWD	5'-(M13F)CTGATGGCCCTGCTTCTCAG-3'	20246	20265
	REV	5'-(M13R)CCTGGCCTGTGTCTTGCCAG-3'	20604	20623
Exon 7	FWD	5'-(M13F)TCTGCAAAGCTCACATTTCC-3'	29892	29911
	REV	5'-(M13R)TATTAAGAGCTAGTGGTGC3'	30184	30203
Exon 8	FWD	5'-(M13F)CAGTGGTCCCAAGTAGTCAC-3'	30741	30760
	FWD	5'-(M13F)AGGAGATAGTGGGGACCC-3'	31207	31226
	REV	5'-(M13R)TAGTTAGTGAGAGCCCTGT-3'	31414	31433

(M13F):5'-TGTAACGACGGCCAGT-3' (M13R):5'-CAGGAAACAGCTATGAC-3'

オキシ法によるダイレクトシーケンスを行った。解析には HITACHI SQ5500E を用いた。塩基配列は, Yoshitake らの報告⁴⁾と比較し, それぞれの変異を確認した。検出した変異は The Haemophilia B Mutation Database-version 12⁹⁾にて既存の報告例の有無につき確認を行った。

⑤未報告例の解析

データベースに登録のなかった Ala28 → Pro, Gln50 → Lys, Leu300 → Pro は, それぞれに対応した制限酵素 *Dpn* I, *Bsm* I (New England Biolabs) による切断にて, 対照者 50 人を解析しその頻度を確認した。制限酵素認識部位に対応しないエクソン 2 の Ala28 → Pro と, イントロン 7 の nt.30156 a → c は, それぞれ人工的に *Cfo* I と *Dde* I 制限部位を導入するため Mutagenic Primer を作製して PCR を行い, 頻度を確認した。

結 果

① FIX:C および FIX:Ag の測定結果と病型

表 2 に FIX:C および FIX:Ag の測定結果を示した。病型の表現型は, 軽症が 3 例, 中等症が

6 例, 重症が 11 例であった。抗原を測定し得た 16 例のうち, 10 例は CRM-reduced 症例であった。またインヒビターを有していた 1 例はハイレスポンド (6BU/ml) であり¹⁰⁾, インヒビター発生前の FIX:C と FIX:Ag はともに 1% 未満の重症であった。

②遺伝子解析

コーディング領域とそのスプライシング部位のダイレクトシーケンスの結果, 20 例から 18 種類の変異を同定した (表 2)。エクソン 2 の Ala28 (gca) → Pro (cca), エクソン 4 の Gln50 (cag) → Lys (aag), エクソン 8 の Leu300 (ctc) → Pro (ccc), イントロン 7 の nt.30156 a → c は未報告の遺伝子異常であった。検出した変異の種類は全て点変異であり, 欠失や挿入などの遺伝子異常は認められなかった。その内訳は 11 種類がミスセンス変異, 4 種類がナンセンス変異, 3 種類がスプライス部位での変異であった。CpG ダイヌクレオチドは 7 例に認められた。これらの変異の部位は, FIX 蛋白質の Gla ドメインに 3 種類, EGF 様ドメインに 4 種類, 活性化ペプチドに 1 種類, カタリティックドメインに 8 種類と, 各ドメインに分散していた。1

表2 Phenotype and genotype analysis of 20 Japanese patients with hemophilia B

No.	FIX:C (%)	FIX:Ag (%)	Phenotype	Location	Nucleotide changed	Mutation
1	<1	<1	CRM negative	Intron 1	6325 g→c	Acceptor splice site (agTT)→(acTT)
2	<1	40	CRM reduced	Exon 2	6424 g→a	E17K
3	<1	50	CRM reduced	Exon 2	6449 t→c	F25S
4	<1	39	CRM reduced	Exon 2	6457 g→c	A28P*
5	7	16	CRM reduced	Exon 4	10400 c→a	Q50K*
6	(<1)	-	-	Exon 5	17692 g→a	G93S
7	(1)	-	-	Exon 5 & 8	17761 c→t & 31218 g→a	R116X & G366E
8	1	15	CRM reduced	Exon 6	20413 c→t	R145C
9	1	32	CRM reduced	Exon 6	20413 c→t	R145C
10	12	12	CRM reduced	Intron 7	30156 a→c	Donor splice site (agGTA)→(agGTC) *
11	3	2	CRM reduced	Intron 7	30818 a→g	Acceptor splice site (atagGT)→(gtagGT)
12	<1	<1	CRM negative	Exon 8	30874 c→t	R252X
13	2	<1	CRM negative	Exon 8	30874 c→t	R252X
14	(4)	-	-	Exon 8	30970 c→g	S283R
15	6	20	CRM reduced	Exon 8	30993 c→t	A291L
16	<1	<1	CRM negative	Exon 8	31020 t→c	L300P*
17	<1	<1	CRM negative	Exon 8	31051 g→a	W310X†
18	<1	2	CRM negative	Exon 8	31119 g→a	R333Q
19	(<1)	-	-	Exon 8	31133 c→t	R338X
20	<1	20	CRM reduced	Exon 8	31218 g→a	G366E

Data in the parenthesis were from medical records.

*: Novel mutation

†: Inhibitor(6 BU/ml)

例からはナンセンス変異 (116Arg → Stop) とミスセンス変異 (366Gly → Glu) を複合変異として検出した。またインヒビターを保有する1例からはナンセンス変異 (310Trp → Stop) を検出した。

③未報告変異の確認

未報告の4つの変異に対しては制限酵素切断法によりその頻度を確認し、変異かポリモルフィズムの判別をした。それぞれの変異は対照者からは1 allele も検出されず、ポリモルフィズムではなく変異であることを確認した。

考 察

現在、The Haemophilia B Mutation Database-version 12⁹⁾ では、2,511 症例の血友病 B の遺伝子異常が登録されており、点変異が2,252 例、30 塩基以下の挿入や欠失が180 例、大きな塩基サイズの挿入や欠失が79 例報告されている。

それらは896 種類の遺伝子異常からなり、F9 の変異の多様性が示されている。その中で、シグナルペプチドやプロタンパク質を含む FIX の461 アミノ酸残基には、523 種類の点変異によるアミノ酸置換が検出されている。本研究のF9 解析においては、20 家系の血友病 B 患者から18 種類の異なる遺伝子異常を同定した。それらは、すべて点変異によるアミノ酸置換であり、欠失や挿入による遺伝子異常は検出されなかった。

Arg116, Arg252, Trp310, Arg338 において、4 種類のナンセンス変異を検出した。一般的にナンセンス変異や挿入・欠失などを有する症例は、異常部位を境に終止もしくはフレームのずれにより蛋白質翻訳が中断し、正常蛋白質が産生されず、重症型になると考えられている。これらの症例は、成熟蛋白質分子が欠損しているために補充療法で投与された FIX を異物認識し、比較的高率に同種抗体であるインヒビター

表 3 Amino acid sequence around Ala28 in the Gla domain of vitamin K-dependent coagulation proteins

	20					28					34				
Factor IX	γ	γ	K	C	S	F	γ	γ	A	R	γ	V	F	γ	N
Factor VII	γ	γ	Q	C	S	F	γ	γ	A	R	γ	I	F	K	D
Prothrombin	γ	γ	T	C	S	Y	γ	γ	A	F	γ	A	L	γ	S
Factor X	γ	γ	T	C	S	Y	γ	γ	A	R	γ	V	F	γ	D
Protein C	γ	γ	I	C	D	F	γ	γ	A	K	γ	I	F	Q	N
Protein S	γ	γ	L	C	N	K	γ	γ	A	R	γ	V	F	γ	N
Protein Z	γ	γ	I	C	V	Y	γ	γ	A	R	γ	V	F	γ	N

Numbers are amino acid residues of Factor IX. γ : γ -carboxyglutamic acid

を発生する可能性がある。本研究 1 例のインヒビター保有症例からは、Trp310 → Stop が検出された。同変異はこれまでに 3 症例^{(11), (12), (13)}が報告されているが、いずれもインヒビターは認められていない。このことより、インヒビター発生の際には当該変異以外にも多様な要因が働くことが推定された。

18 種類の遺伝子異常のうち、4 つの新しい変異を同定した。エクソン 2 の Ala28 → Pro は、Gla ドメイン内で検出された。データベース⁹⁾には Ala28 における他のアミノ酸置換症例の報告もない。また、ビタミン K 依存性凝固因子間で Ala28 は保存性が高いが (表 3)、第 VII 因子、第 X 因子、プロテイン C の先天性欠乏症での遺伝子解析例においても、Ala28 に対応するアミノ酸の点変異の報告例はない。患者の FIX:C は 1.0% 未満であり、かつ FIX:Ag は 39% であったことから、この変異の表現型は CRM-reduced であった。これらより、Ala28 のイミノ酸 Pro への置換は、Gla ドメイン高次構造を不安定化させることにより、結果として機能障害をきたすことが考えられた。

エクソン 4 の Gln50 → Lys は、FIX 蛋白質の EGF-1 ドメイン内のミスセンス変異であった。EGF-1 ドメインの点変異は、データベース⁹⁾には 179 例が登録されている。そのうち Gln50 では、His と Phe へのミスセンス変異とナンセンス変異が計 7 例報告されている。ナンセンス変異と Phe のミスセンス変異はそれぞれ重症型であったが、His 置換での 2 例は軽症例が報告されている。Spritzer ら¹⁴⁾は、FIX の EGF-1

ドメインが逆平行 β シート構造と β ターンからなり、Asp47, Asp49, Asp64 の各残基が Ca^{2+} 結合部位を構成していることを推定した。その後、Rao ら¹⁵⁾は、Gln50, Asp47, Gly48, Asp65 が 1 分子の Ca^{2+} イオンと配位している事を EGF-1 ドメイン結晶構造で明らかにした。このことから、Gln50 → Lys 変異により正常な Ca^{2+} 結合能が失われ、さらに近傍の立体構造変化にも影響を与えることが推測された。

イントロン 7 + 3 のドナースプライス部位の変異は、a → c 変異により正常なスプライス機構が障害され、イントロン 7 の開始地点から 374bp 下流にある潜在的スプライス部位が活性化する、または、エクソンスキッピングが起き異常 mRNA が形成されると考えられた。通常不安定な mRNA は RNA 監視機構^{(16), (17)}によって生体内で速やかに分解されるが、しかし、この患者では FIX:C と FIX:Ag はそれぞれ 12% であった。このことは、一部は正常なスプライシング機構が働いていることを推測させた。

今回の血友病 B の遺伝子解析では、遺伝子型と血漿レベルによる表現型の関連を中心に検討した。血友病 B の F9 解析は世界各国で進められており、既に 2,500 例以上の患者の遺伝子異常が同定されている。本研究は 20 例という比較的少数検体を用いた解析であるが、18 種類中 4 種類の新しい変異を検出した。このことは、日本人の F9 の変異に特有の多様性がある可能性も示唆させるものであった。血友病 B 患者の個々の症例において、F9 の遺伝子型を把握することは、ナンセンス変異、欠失、挿入

などに起因する重症例における高率なインヒビター出現を考慮し、慎重に補充療法を進めるうえで重要である。また、今後本邦でも行なわれるであろう遺伝子治療の臨床応用に向け、表現型と同様に遺伝子型の把握は基礎データとして不可欠である。

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Eighteen point mutations in Japanese patients with hemophilia B

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Key words: Hemophilia B, Factor IX gene, Nucleotide sequence, Point mutation, Genotype

Hemophilia B is a hereditary bleeding disorder caused by quantitative or qualitative abnormalities in coagulation factor IX. To determine the molecular defects in 20 Japanese patients from 20 families, we amplified and sequenced all the exons, their splice junctions, and the 5' flanking region of the factor IX gene. Eighteen different mutations (11 missense, 4 nonsense, and 3 splice site mutations) were identified in samples from the 20 patients. No deletion or insertion mutation was detected. Four mutations, Ala28 → Pro, Gln50 → Lys, Leu300 → Pro, and A → C transversion at the donor splice site of intron 7, have not been reported previously. To date, 896 genetic defects have been registered in the international hemophilia B mutation database. These findings suggest that more hemophilia B mutations are to be identified in the future.

Frequent Transmission of Cytotoxic-T-Lymphocyte Escape Mutants of Human Immunodeficiency Virus Type 1 in the Highly HLA-A24-Positive Japanese Population

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Although Japan is classified as a country with a low prevalence of human immunodeficiency virus type 1 (HIV-1), domestic sexual transmission has been increasing steadily. Because 70% of the Japanese population expresses HLA-A24 (genotype HLA-A*2402), we wished to assess the effect of the dominant HLA type on the evolution and transmission of HIV-1 among the Japanese population. Twenty-three out of 25 A24-positive Japanese patients had a Y-to-F substitution at the second position [Nef138-10(2F)] in an immunodominant A24-restricted CTL epitope in their HIV-1 *nef* gene (Nef138-10). None of 12 A24-negative Japanese hemophiliacs but 9 out of 16 patients infected through unprotected sexual intercourse had Nef138-10(2F) ($P < 0.01$). Two of two A24-positive but none of six A24-negative Australians had Nef138-10(2F). Nef138-10(2F) peptides bound well to the HLA-A*2402 heavy chain; however, Nef138-10(2F) was expressed poorly on the cell surface from the native protein. Thus, HIV-1 with Nef138-10(2F) appears to be a cytotoxic-T-lymphocyte escape mutant and has been transmitted frequently by sexual contact among the highly A24-positive Japanese population.

While cytotoxic T lymphocytes (CTLs) exert immune pressure on human immunodeficiency virus type 1 (HIV-1) throughout the course of primary and chronic infection (4, 24, 30), HIV-1 escapes through a variety of immune evading mechanisms such as downregulation of HLA class I molecules by Nef (7, 32, 33, 36) and defects in differentiation and maturation of CTLs (2, 6, 27, 35). Viral mutation also plays a crucial role in immune escape, and CTL escape mutant viruses may appear early or late in the clinical course of infection (5, 14, 31). Mutations leading to CTL escape may occur at amino acid residues essential for major histocompatibility complex binding (8), for T-cell-receptor recognition (10), or in flanking regions that affect antigen processing (3, 26).

HIV-1 CTL escape mutants may be stable. One such example at the HLA-B27-restricted Gag epitope, which is related to slower disease progression in adults, could be transmitted vertically from mother to child (12). Although significant association between HLA alleles and polymorphism in reverse transcriptase sequences in a large cohort of patients indicated HIV-1 adaptation at a population level (28), direct horizontal transmission of CTL escape mutants is yet to be shown.

Japan is classified as a country of low HIV-1 prevalence; however, national HIV-1 and AIDS surveillance has shown a steady increase of HIV-1 and AIDS cases mainly through un-

protected sexual intercourse (USI) (84% of HIV-1 patients and 71% of AIDS patients were infected through USI within the country) (1). The Japanese population is less polymorphic than other populations in that 70% express HLA-A24 (genotype HLA-A*2402) (13). We speculated that stable CTL escape mutants from HLA-A24 might be transmitted more frequently in Japan than in other countries where the prevalence of HLA-A24 is much lower. We postulated that Japanese hemophiliacs with HIV-1 infection might be a good comparator group since they were infected directly by contaminated blood products from abroad. We therefore examined an immunodominant CTL epitope in the *nef* gene (Nef138-10) in HLA-A24-positive and -negative hemophiliacs and compared the sequence with sequences from those patients infected through USI (13, 18). We included Caucasian Australians infected through USI as another control of transmission of CTL escape mutants in a country where HLA-A24 is less prevalent (19).

MATERIALS AND METHODS

Patient samples. For sequence analysis, blood specimens were collected in EDTA. Plasmas were separated and preserved at -80°C until use. For enzyme-linked immunospot (ELISPOT) assay, peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood and used on the day of the assay. Patient HLA was typed serologically. In selected patients, HLA genotype was determined after written informed consent was obtained. The study was approved by institutional review boards. All patients serologically typed as A24 positive proved to be positive for HLA-A*2402.

RNA extraction and reverse transcription. Viral RNA was extracted from 140 μl of plasma from patients by using the QIAamp viral RNA Mini kit (QIAGEN) and subjected to reverse transcription according to the manufacturer's protocol with SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) and 5 μM random primers (Takara).

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PCR amplification and sequencing. Fifteen microliters of cDNA (a one-sixth volume of the reverse transcription reaction) was subjected to the first PCR. One-tenth of the first PCR was subjected to the nested PCR. PCR was performed by using Ex-Taq (Takara) with 35 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and a final extension for 7 min at 72°C. The primer sets are as follows (all nucleotide positions are in accordance with the HIV-1 SF2 strain). For the *env* V3 region, first PCR primer set 1, primers CBE297P (5'-GGTAGAACAG ATGCATGAGGAT-3') (consensus B *env*, nucleotides [nt] 297 to 318) and E7668 M (5'-TTCTCCAATTGTCCTCATATCTCCTCCTCCA-3') (SF2, nt 7668 to 7636) were used; and for the second PCR primer set 1, primers E6554P (5'-ATCAGTTTATGGGATCAAAGCC-3') (SF2, nt 6554 to 6575) and E7353 M (5'-ACAATTTCTGGGTCCCTCCTGAGGA-3') (SF2, nt 7353 to 7328) were used. For the first PCR primer set 2, primers E6984P (5'-ACATGGAAT TAGGCCA-3') (SF2, nt 6984 to 7000) and E7395 M (5'-TTACAGTAGAAA AATTCCC-3') (SF2, nt 7395 to 7375) were used; and for the second PCR primer set 2, primers E7028P (5'-GGCAGTCTAGCAGAAGAAGA-3') (SF2, nt 7028 to 7047) and E7353 M (5'-ACAATTTCTGGGTCCCTCCTGAGGA-3') (SF2, nt 7353 to 7328) were used. For the first PCR primer set 3, primers P6951 (5'-GACCATGTACAATGTACG-3') (SF2, nt 6951 to 6970) and M7592 (5'-CTCTTGTTAATAGCAGCCCT-3') (SF2, nt 7592 to 7573) were used; and for the second PCR primer set 3, primers E6984P (5'-ACATGGAAT TTAGGCCA-3') (SF2, nt 6984 to 7000) and E7353 M (5'-ACAATTTCTGGG TCCCTCCTGAGGA-3') (SF2, nt 7353 to 7328) were used.

For the Nef138-10 epitope, first PCR primer set 1, primers n226p (5'-CTCA GGTACCTTTAAGACCAATG-3') (nt 9028 to 9050) and n650m (5'-GAAAG TCCCCAGCGGAAAGTCCC-3') (nt 9474 to 9452) were used; and for the second PCR primer set 1, primers n296p (5'-GGGACTGGAAGGGCTAATT TGGT-3') (nt 9098 to 9120) and n564m (5'-GAAATGCTAGTTTGCTGTCA AAC-3') (nt 9387 to 9365) were used. For the first PCR primer set 2, primers P8923 (5'-TGAAAAACATGGAGCAATCA-3') (nt 8923 to 8944) and M9290 (5'-TCCTTCATTGGCCTCTTCTAC-3') (nt 9290 to 9270) were used; and for the second PCR primer set 2, primers P8924 (5'-GGAAAAACATGGAGCAA TCAC-3') (nt 8924 to 8945) and M9288 (5'-CTTCATTGGCCTCTTCTACCT-3') (nt 9288 to 9268) were used. For the first PCR primer set 3, primers P8923 (5'-TGAAAAACATGGAGCAATCA-3') (nt 8923 to 8944) and n694m (5'-C AGCATCTGAGGGGACGCCA-3') (nt 9525 to 9506) were used; and for the second PCR primer set 3, primers n226p (5'-CTCAGGTACCTTTAAGACCA ATG-3') (nt 9028 to 9050) and n532m (5'-TCTCCGCGTCTCCATCCA-3') (nt 9345 to 9326) were used.

The PCR products were electrophoresed through agarose gels and purified with a Minielute gel extraction kit (QIAGEN) before sequencing. Purified PCR products were directly sequenced. When sequence ambiguities resulted, DNA fragments were subcloned into the pGEM-T vector (Promega) and sequenced. DNA sequencing was performed by using an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems) on a Perkin-Elmer ABI-377 sequencer.

Cells and media. T2-A24, a kind gift from K. Kuzushima, was cultured in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma) and 0.8 mg of G418 (Invitrogen)/ml (25). We transformed PBMCs from an HLA-A*2402-positive person with human T-cell leukemia virus type 1 (HTLV-1) and established an HLA-A*2402- and CD4-positive-T-cell line (KWN-T4). KWN-T4 was cultured with RPMI 1640 supplemented with 25 U of interleukin-2 (Wako)/ml, 100 U of penicillin/ml, 100 U of streptomycin (Invitrogen)/ml, and 10% heat-inactivated FCS (JRH Bioscience). We also established Nef138-10-specific CTL clones as previously described (22). CTL clones were cultured with RPMI 1640 supplemented with 50 U of interleukin-2/ml, 100 U of penicillin/ml, 100 U of streptomycin/ml, and 10% heat-inactivated FCS.

Peptides. Synthetic peptides Nef138-10 (RYPLTFGWCF), 2F (RFPLTFGW CF), 5C (RYPLCFGWCF), and 2F5C (RFPLCFGWCF) were purchased from Sigma-Genosys. All peptides were more than 95% pure as determined by high-performance liquid chromatography and mass spectroscopy.

Peptide binding assays. Peptide binding to HLA-A*2402 was quantified by using a T2-A24 stabilization assay as previously described (25). T2-A24 cells were incubated at 26°C for 16 h, and then 2×10^5 cells were incubated with peptides at concentrations from 10^{-4} to 10^{-9} M for 1 h at 4°C. After incubation for 3 h at 37°C, the cells were stained with anti-HLA-A24 monoclonal antibody, A11.1 M (11), and an R-phycoerythrin (RPE)-conjugated F(ab')₂ fragment of anti-mouse immunoglobulin (DAKO). The mean fluorescence intensity was measured by FACSCalibur (Becton Dickinson).

ELISPOT assay and functional avidity analysis. Freshly prepared PBMCs (20,000 to 50,000) were added to 96-well multiscreen plates (Millipore) which had been precoated with 100 μ l of 5 μ g of anti-gamma interferon (IFN- γ) monoclonal antibody 1-D1K (Mabtech)/ml at room temperature for 3 h and

blocked with RPMI 1640 medium containing 10% FCS for 1 h. The cells were cultured with synthetic peptide Nef138-10 or its derivatives at concentrations from 10^{-5} to 10^{-11} M for 18 h. After the plates were washed, 100 μ l of 1 μ g of biotinylated anti-IFN- γ monoclonal antibody 7-B6-1 (Mabtech)/ml was added and incubated at room temperature for 90 min. After the plates were washed again, 100 μ l of 1:1,000-diluted streptavidin-alkaline phosphatase conjugate (Mabtech) was added and incubated at room temperature for 60 min. Spots were developed with an alkaline phosphatase conjugate substrate kit (Bio-Rad) and counted with a KS ELISPOT compact (Carl Zeiss). The IFN- γ responses to peptide dilutions were expressed as a percentage of the maximal IFN- γ response seen in each individual assay.

Expression of recombinant Nef protein. Mutations were introduced into *nef* derived from HIV-1 strain SF2 by site-directed mutagenesis based on overlap extension (16). Four proline residues in the Nef proline-rich domain that are important for HLA class I down-regulation were replaced by alanine as described previously (36). The wild type and various *nef* mutants were tagged by His₆ and introduced into a Sendai virus vector (SeV) as previously described (36). For Western blot analysis, KWN-T4 cells were infected with various SeVs at a multiplicity of infection of 10 and lysed 20 h after infection. Western blot analysis was performed according to the standard procedure. Anti-His₆ antibody (QIAGEN) and anti-SeV mouse antiserum were used to detect Nef and SeV proteins, respectively.

⁵¹Cr release assay. Cytotoxicity was measured with a standard ⁵¹Cr release assay as previously described (21). Briefly, KWN-T4 was labeled with 100 μ Ci of Na⁵¹CrO₄ for 2 h and washed three times with R10. Labeled cells (2×10^5) were added to a 96-well round-bottom microtiter plate with a corresponding amount of peptide. After 1 h of incubation, Nef138-10-specific CTL clones were added and incubated for 4 h. When SeV-infected cells were used as target cells, the cells were infected with SeVs at a multiplicity of infection of 10, 20 h before adding the CTLs.

The supernatants were collected and analyzed with a microbeta counter. Spontaneous ⁵¹Cr release was determined by measuring counts per minute in the supernatant of wells containing only target cells (cpm_{spn}). The maximum release (cpm_{max}) was determined by measuring the release of ⁵¹Cr from target cells in the presence of 2% Triton X-100. Specific lysis was determined as follows: specific lysis = (cpm_{exp} - cpm_{spn})/(cpm_{max} - cpm_{spn}) \times 100, where cpm_{exp} represents the counts per minute in the supernatant of wells containing target and effector cells.

RESULTS

Sexual transmission of HIV-1 with stereotypic amino acid substitution among the Japanese population. Only patients infected with virus subtyped as B by phylogenetic comparison of envelope sequences were included to avoid potential bias introduced by sequence differences across subtypes (data not shown). We extensively sequenced the Nef138-10 epitope and its flanking region from plasma HIV-1 RNA of 23 Japanese hemophiliacs (11 A24-positive and 12 A24-negative individuals) and 30 Japanese (14 A24-positive and 16 A24-negative individuals) and 8 Caucasian Australians (2 A24-positive and 6 A24-negative individuals) infected through USI (Table 1). Ten out of 11 A24-positive but none of A24-negative Japanese hemophiliacs had a Y-to-F amino acid substitution at the second position [Nef138-10(2F)] (Fig. 1A) ($P < 0.01$), suggesting that HLA-A24 selected for Nef138-10(2F). In the case of patients infected through USI, 13 out of 14 A24-positive and 9 out of 16 A24-negative Japanese patients had Nef138-10(2F) by direct sequencing (Fig. 1B) (data not significant). The frequency of Nef138-10(2F) was significantly higher in Japanese A24-negative patients infected through USI than A24-negative hemophiliacs ($P < 0.01$). Two out of two A24-positive but none of six A24-negative Caucasian Australians had Nef138-10(2F) (Fig. 1C). The frequency of Nef138-10(2F) in A24-negative patients infected through USI was significantly higher for Japanese patients than for Australian patients ($P < 0.05$), suggesting that sexual transmission of the variant was more

TABLE 1. Patient profile^a

Patient ID	Sex	HLA type	No. of CD4 cells/ μ l	Viral load (copies/ml)	Sample date (mo/day/yr)	HIV subtype
A24-positive Japanese hemophiliacs						
A24-J037	M	A24/26, B35/51	207	180,000 ^b	03/09/95	B
A24-J041	M	A24/26, B44/61	261	7,500 ^{b,d}	03/09/95	B
A24-J033	M	A24/26, B46/52	27	200,000 ^b	03/27/95	B
A24-J035	M	A24, B40/48	148	360,000	04/10/95	B
A24-J031	M	A24/31, B51/60	29	180,000 ^b	10/23/95	B
A24-J030	M	A11/24, B13/62	3	380,000 ^{b,d}	02/26/96	B
A24-J029	M	A24/31, B35/61	38	ND	04/01/96	B
A24-J036	M	A2 /24, B35/51	60	74,000 ^b	05/13/96	B
A24-J034	M	A24, B46/52	180	74,000 ^{b,d}	05/20/96	B
A24-J038	M	A2 /24, B51/62	356	29,000 ^b	03/03/97	B
A24-J005	M	A24, B52/70	39	220,000 ^b	06/19/97	B
A24-negative Japanese hemophiliacs						
NA24-J037	M	A26, B40	8	>1,600,000 ^{b,d}	06/08/95	B
NA24-J035	M	A11/26, B54/56	342	100,000 ^b	09/07/95	B
NA24-J031	M	A2/26, B51/61	521	130,000 ^b	09/18/95	B
NA24-J041	M	A26, B39/54	12	700,000 ^{b,d}	10/05/95	B
NA24-J032	M	A2/11, B46/54	1 ^d	150,000 ^b	11/10/95	ND
NA24-J030	M	A31/33, B44/51	363	65,000 ^b	03/21/96	B
NA24-J040	M	A2/33, B17/54	101	74,000 ^b	03/21/96	ND
NA24-J033	M	A26, B61	143	140,000 ^b	04/18/96	B
NA24-J029	M	A11/33, B44/51	401	<10,000	07/15/96	B
NA24-J034	M	A11/33, B17/56	38	81,000 ^b	08/15/96	B
NA24-J039	M	A11/26, B51/62	3	88,000 ^b	09/01/97	B
NA24-J006	M	A2/26, B39/61	335	9,200	10/30/00	B
A24-positive Japanese infected through USI						
A24-J006	M	A2/24, B7/54	212	33,000	09/19/97	B
A24-J007	M	A24/26, B17/56	103	120,000	11/06/97	B
A24-J009	M	A24, B48/52	278	4,500	01/19/98	B
A24-J010	M	A24, B52	393	18,000	03/09/98	B
A24-J024	M	A24, B35/61	274	110,000	10/27/98	B
A24-J012	M	A24/26, B46/60	253	24,000	07/19/99	B
A24-J013	M	A24/26, B35/48	168	15,000	9/20/99	B
A24-J016	M	A11/24, B7/55	245	150,000	05/15/00	B
A24-J017	M	A1/24, B54/70	255	70,000	10/17/00	B
A24-J018	M	A24/31, B37/61	185	8,300	01/04/01	B
A24-J025	M	A24, B51/52	282	130,000	06/07/01	B
A24-J023	M	A2/24, B51/54	856 ^d	17,000 ^d	08/06/01	B
A24-J021	M	A2/24, B46/52	344	35,000	11/26/01	B
A24-J026	M	A2/24, B13/51	381	110,000	11/28/01	B
A24-negative Japanese infected through USI						
NA24-J025	M	A2/31, B51/61	352	18,000 ^b	03/23/95	B
NA24-J023	M	A11/26, B35/51	23	5,000 ^b	04/01/96	ND
NA24-J021	M	A26, B52/54	9	44,000	08/04/97	B
NA24-J018	M	A2, B39/60	378	72,000	04/06/98	B
NA24-J017	M	A11/31, B51/56	197	72,000	04/16/98	B
NA24-J016	M	A3/31, B51/58	257	200,000	05/25/98	B
NA24-J015	M	A2/26, B51/62	543	13,000	06/26/98	B
NA24-J012	M	A31, B13/51	268	26,000	10/19/98	B
NA24-J011	M	A2, B55/60	408	12,000	10/22/98	B
NA24-J010	M	A2/26, B51/61	206	16,000	12/17/98	B
NA24-J009	M	A2, B52/60	115	850,000	05/24/99	B
NA24-J008	M	A11/33, B44/60	312	2,600	07/08/99	ND
NA24-J007	M	A26, B7/52	396	450	08/09/00	B
NA24-J005	M	A2/31, B48/52	604	17,000	01/18/01	B
NA24-J003	M	A31/33, B44/51	308	20,000	06/04/01	B
NA24-J002	M	A2/33, B44/46	496	14,000	09/27/01	ND
A24 positive Australian infected through USI						
A24-A001	M	A3/24, B7	255	38,000	08/16/96	ND
A24-A002	M	A24/30, B13	598	21,700	03/22/01	B
A24-negative Australian infected through USI						
NA24-A007	M	A2/3, B7	704	ND ^c	11/02/95	B
NA24-A005	M	A1/3, B8/70	620	7,700	05/26/96	B
NA24-A013	M	A32, B13/64	851	23,200	09/28/98	B
NA24-A008	M	A2/3, B39/44	543	52,836	01/04/99	B
NA24-A003	M	A2, B18/62	575	19,400	11/06/99	B
NA24-A006	M	A3/26, B18/27	594	18,200	04/13/00	B

^a ND, not determined.^b Data were obtained by Branch DNATM version 1.0.^c Nearest data were 17,000 with CD4 counts of 638.^d Nearest data were within 6 months of sample collection.

A

A24-positive Japanese hemophiliacs				A24-negative Japanese hemophiliacs			
	flanking	CTL epitope	flanking		flanking	CTL epitope	flanking
Patient ID	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV	Patient ID	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV
A24-J041	-----V	-F-----	-----M	NA24-J037	-----	-----	-----
A24-J033	---E---T	-F-----Y	-----D--	NA24-J035	-----	-----	---M---
A24-J031	-H-----T	-F-----	-----	NA24-J031	-----	-----	---G/E-V/1
A24-J030	-----T	-F-C----	-----	NA24-J041	-----	-----	-----DE
A24-J034	-----T	-F-----	-----DQ-Q-	NA24-J032	-----	-----	---M---
A24-J038	-----T	---C----	-----D-D--	NA24-J030	--S-----V	---C----	-----
A24-J005	-D/E-----T	-F-----	-----	NA24-J040	-----	-----	-----I
A24-J029	-----V/T	-F-----	-----Q-	NA24-J033	-----	-----	-L/V-----
A24-J037	--C-----T	-F-----	-----	NA24-J029	-H-----	-----	-----D-
A24-J035	-----T	-F-----	-----	NA24-J034	-----	-----	---V/L---
A24-J036	--C-----T	-F-----	-----	NA24-J039	-----	---C----	---D-D--
				NA24-J006	-----V	---C----	---E---

B

A24-positive Japanese infected through USI				A24-negative Japanese infected through USI			
	flanking	CTL epitope	flanking		flanking	CTL epitope	flanking
Patient ID	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV	Patient ID	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV
A24-J006	-----V	-F-----	---E/D--Q-	NA24-J025	-H-----V	---C----	---D-D/AQ-
A24-J007	-----T	-F-C----	---A--E-	NA24-J023*	-----T	-Y/W/F-----	---I Y 2/11, F 3/11, W 5/11
A24-J009	-----T	-F-----	-----	NA24-J021	-----	-----	---N-Q-
A24-J010	-----T	-F-----	---QR-	NA24-J018*	-----T	-Y/F--C----	Y 8/10, F 2/10
A24-J012	-----T	-F-----	---D-	NA24-J017*	-----T	-Y/F-----	---L- Y 5/13, F 8/13
A24-J013	-----T	-F-----	---D-DQ-	NA24-J016	-----V	-----	---L-Q-
A24-J016	-D-----V	---C----	---DQD--	NA24-J015	-----T	-F-----	---D-DO-
A24-J017	-D-----T	-F-C----	-----I	NA24-J012	-H/QS-----T	-----	---D-DO-
A24-J018	-----T	-F-----	---I	NA24-J011	-----T	-F-----	---NO-
A24-J023	-----T	-F-----	---L-GEA	NA24-J010	-----	-----	-----
A24-J021	-----T	-F-----	---D-DQ-	NA24-J009	-----T	-F-----	---NO-
A24-J024	-----T	-F-----	---D-D-	NA24-J008	-D-----T	-F-----	---L--Q-
A24-J025	-D-----T	-F-----	---DQDQ-	NA24-J007	-----T	-F-----	---NO-
A24-J026	-----T	-F-----	---KQ-	NA24-J005	-G/D-----T	-F-----	---DQDQ-
				NA24-J003	-H-----	-----	---DQ--
				NA24-J002	-Q/HG-----	-----	---D-DQ-

C

A24-positive Australian infected through USI				A24-negative Australian infected through USI			
	flanking	CTL epitope	flanking		flanking	CTL epitope	flanking
Patient ID	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV	Patient ID	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV
A24-A001	-----T	-F-----	-----	NA24-A007	-----V	-----	-----
A24-A002	-----T	-F-----	---M-----	NA24-A005	-----V	-----	-----
				NA24-A013	-----	-----	-----
				NA24-A008	-H-----	-----	---M-P/O--
				NA24-A003	-H-----	-----	---D-D-
				NA24-A006	-----	---C----	---E---

FIG. 1. Nef138-10 epitope and its flanking sequences. Amino acid sequences deduced from the direct DNA sequencing of Nef138-10 CTL epitope and both flanking regions are presented. Wild-type sequences (HIV-1 strain SF2) are presented on the top. Dashes indicate the same amino acid as that of the wild type. Sequence substitutions are presented by single amino acid characters. Where a mixture of two or three amino acids was plausible, two or three amino acids were shown together separated by a slash. A single dash indicates that the sequences could not be determined by ambiguities. (A) Sequences from A24-positive and -negative Japanese hemophiliacs. (B) Sequences from A24-positive and -negative Japanese patients infected through USI. Asterisks indicate samples for which sequence ambiguities were found by direct sequence analysis. We cloned these PCR fragments into the pGEM-T vector and sequenced each 10 to 13 colonies. All amino acid sequences are indicated. (C) Sequences from A24-positive and -negative Australians infected through USI.

frequent among the Japanese population, which has a higher prevalence of HLA-A*2402.

Nef138-10(2F) accompanied a particular amino acid substitution in the N-terminal flanking region. We detected an I-to-T substitution at the -1 position (-1T) in 32 flanking sequences out of 34 accompanying Nef138-10(2F) sequences (94%), while others were two I-to-V substitutions (Fig. 1). The -1T substitution was quite unusual in the flanking region of the wild-type Nef138-10 CTL epitope in our cohort (Fig. 1).

Reversion of CTL escape mutants. Since three acutely infected A24-positive patients (A24-J023, A24-J024, and A24-J025) had Nef138-10(2F) in their earliest plasma samples available, we could not demonstrate the evolution of Nef138-10(2F) from the wild type under the selective pressure of HLA-A*2402 (data not shown). However, we could analyze serial samples from chronically infected A24-negative patients who had been followed without treatment over years. All the 12 cloned sequences in the earliest plasma samples available from NA24-J015 had F at the second position; however, 11 out of 11 clones displayed wild-type sequence within a year (Fig. 2A). It is interesting that the -1T substitution in the flanking region

was present for at least a further two years before reverting to the wild type (Fig. 2A). In another chronically infected A24-negative patient (NA24-J018), we observed that the proportion of Nef138-10(2F) decreased gradually but persisted in the plasma for almost two years after the start of the follow-up (Fig. 2B). This patient had a T-to-C substitution at the fifth position with [Nef138-10(2F5C)] or without [Nef138-10(5C)] a substitution at the second position (Fig. 2B). Interestingly, the ratio of Nef138-10(2F5C) to Nef138-10(5C) decreased as time went by (Fig. 2B), suggesting that Nef138-10(5C) is more stable than Nef138-10(2F5C). Actually, we observed Nef138-10(5C) in both A24-positive and -negative patients (Fig. 1).

In order to elucidate the higher stability of the 5C rather than the 2F substitution, we examined the codon usage at these positions (data not shown). The wild-type codon for the second tyrosine (Y) residue in Nef138-10 was coded by TAT or TAC in 23 (77%) and 12 (40%) out of 30 patients, respectively. Five patients (17%) had a mixture of TAT and TAC for the codon (data not shown). Mutated nucleotide triplet TTT or TTC was responsible for the Y-to-F amino acid substitution in 27 (80%) and 9 (26%) out of 34 patients, respectively (data not shown).

A

Patient ID	Sample Date		nef138-10			cloning
	M/D/Y	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV		
NA24-J015	06/26/98	-----T	-F-----	-----D-DQ-	direct	
		-----T	-F-----	-----D-DQ-	11/12	
		-----T	-F----R--	-----D-DQ-	1/12	
NA24-J015	06/07/99	-----T	-Y-----	-----D-DQ-	direct	
		-----T	-Y-----	-----D-DQ-	9/11	
		-R-----T	-Y-----	-----D-DQ-	1/11	
		-----T	-Y-----	--I--D-DQ-	1/11	
NA24-J015	03/09/00	-----T	-Y-----	-----D-DQ-	direct	
NA24-J015	04/16/01	-----T	-Y-----	-----D-DQ-	direct	
NA24-J015	01/16/03	-----	-Y-----	-----D-DQ-	direct	
		-----	-Y-----	-----D-DQ-	10/10	

B

Patient ID	Sample Date		nef138-10			cloning
	M/D/Y	WQNYTPGPGI	RYELTFGWCF	KLVPVEPEKV		
NA24-J018	04/08/96	-----T	-F--C----	-----Q-	direct	
		-----T	-F--C----	-----Q-	7/11	
		-----P	-F--C----	-----Q-	3/11	
		-----P	-Y--C----	-----Q-	1/11	
NA24-J018	06/02/97	-----T	-F/Y--C----	-----Q-	direct	
		-----T	-F--C----	-----Q-	7/13	
		-----T	-Y--C----	-----Q-	3/13	
		-----A	-Y--C----	-----Q-	1/13	
NA24-J018	04/06/98	-----T	-Y/F--C----	-----	direct	
		-----T	-Y--C----	-----Q-	6/10	
		-----T	-F--C----	-----Q-	2/10	
		-----T	-Y--C----	-----	2/10	

FIG. 2. Serial Nef138-10 epitope and its flanking sequences in two A24-negative patients. Data are shown as described in the legend to Fig. 1. Fractional numbers in the right-most column indicate clone numbers with the sequences shown in the numerator and total clone numbers sequenced shown in denominator. "Direct" indicates the result of direct sequencing. (A) Patient NA24-Jo15. (B) Patient NA24-J018.

In two patients (6%) Nef138-10(2F) was coded by a mixture of HIV-1 using TTT and TTC for the codon. It appeared that at least one point mutation was necessary for the Y-to-F amino acid substitution. The wild-type codon for the fifth threonine (T) residue in Nef138-10 was coded by ACC or ACT in 49 (98%) and 2 (4%) out of 50 patients. One patient (2%) had a mixture of ACC and ACT. Mutated nucleotide triplet TGC or TGT was responsible for the T-to-C amino acid substitution in 5 (45%) and 6 (55%) out of 11 patients, respectively (data not shown). It appeared that at least two nucleotides had to be mutated for the T-to-C amino acid substitution, although we could not exclude the possibility of a three-nucleotide mutation for the amino acid substitution. Therefore, a Y-to-F amino acid substitution, or vice versa, at the second position required less nucleotide mutations than did the T-to-C substitution at the fifth position.

Peptide-based analysis of Nef138-10 and its variants. We measured the binding affinities of Nef138-10 and its variants to HLA-A*2402 (Fig. 3). Although a Y-to-F substitution occurred at the amino acid crucial for peptide affinity with the binding groove, Nef138-10(2F) binding to the HLA-A*2402 heavy chain was essentially preserved. However, the acquisition of a T-to-C substitution at the fifth position, such as Nef138-10(2F5C) and Nef138-10(5C), greatly reduced the binding affinity (Fig. 3). A functional avidity assay in which PBMCs from five patients were used confirmed the results of the binding assay (Fig. 4). Namely, the patients' PBMCs re-

cognized Nef138-10(2F) at a very low concentration (one-half maximum response <1 nM) and had equivalent or even higher functional avidity than did the wild-type peptide. On the contrary, patients' PBMCs showed very low functional avidity against Nef138-10(2F5C) and Nef138-10(5C) (one-half maximum response >100 nM).

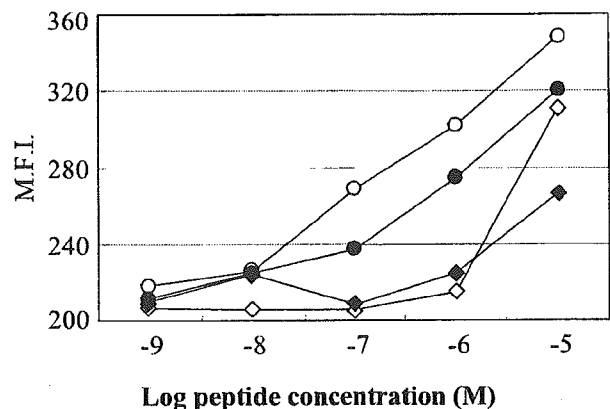


FIG. 3. Binding of the wild-type and mutant peptides to HLA-A*2402 molecules. Peptide binding to HLA-A*2402 was quantified by using a T2-A24 stabilization assay. Symbols: ○, wild type; ●, 2F; ◇, 5C; ◆, 2F5C. M.F.I., mean fluorescence intensity.