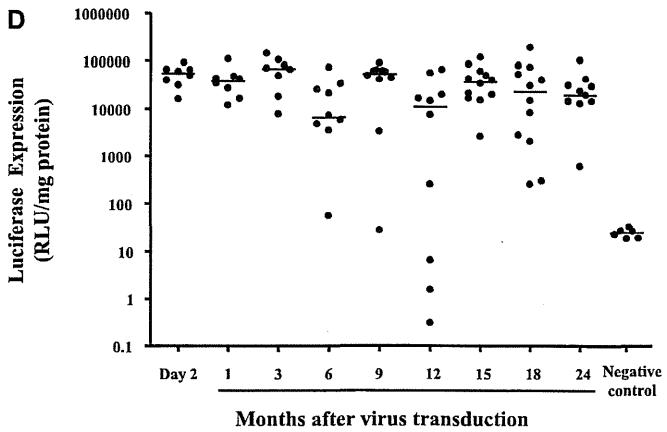


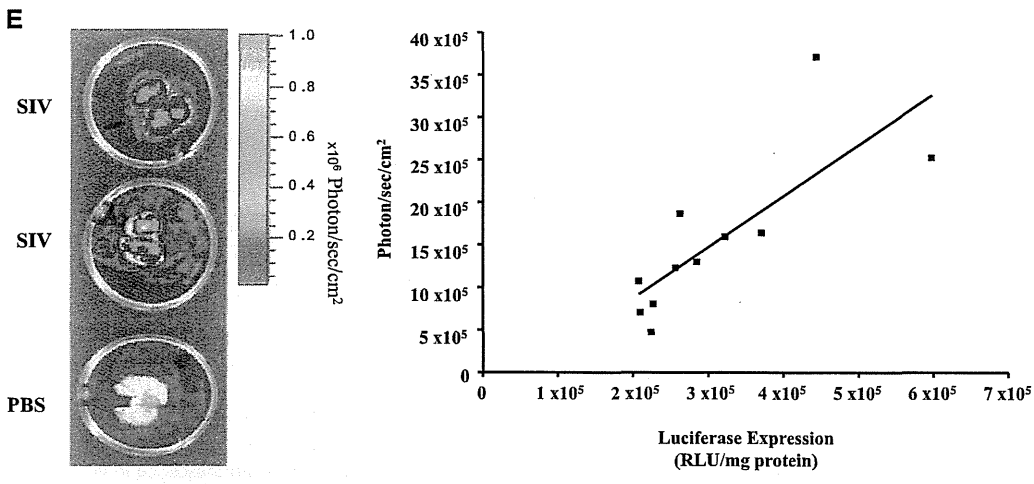
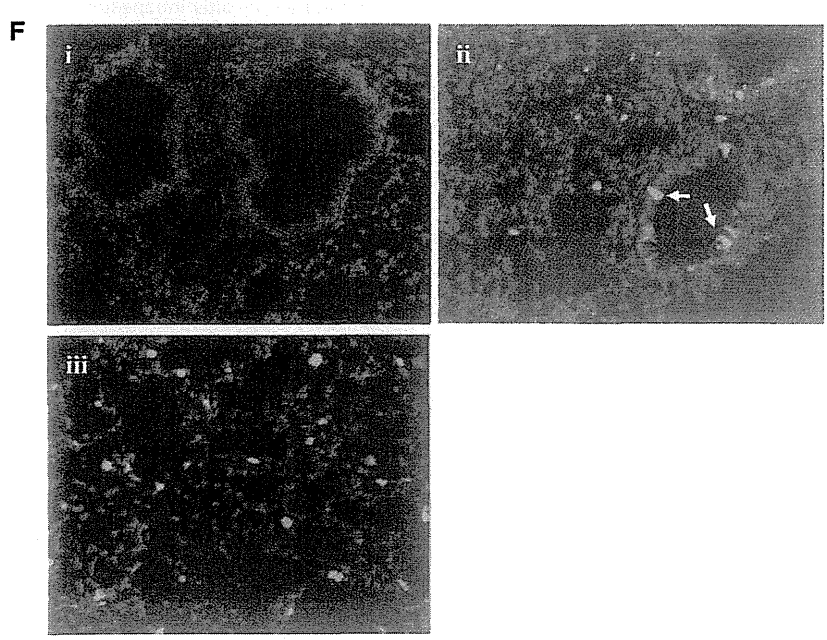


Figure 1. (*Continued*).



administration of the viral vector is feasible and if this leads to incrementally increased gene expression compared with a single dose. Mice (n = 8 per group) were treated with either F/HN-SIV-Lux daily for 10 days (10^6 TU/day in 100 μl) or with PBS (100 μl) for 5 days followed by 5 days of F/HN-SIV-Lux (10^6 TU/day) or with D-PBS for 9 days followed by 1 day of F/HN-SIV-Lux (10^6 TU/day); Lux expression was quantified 28 days after the last dose. Gene expression was significantly correlated (Pearson $r^2 = 0.61$; $P > 0.0001$) with the number of F/HN-SIV-Lux doses given (Figure 3A) and indicated that daily repeat

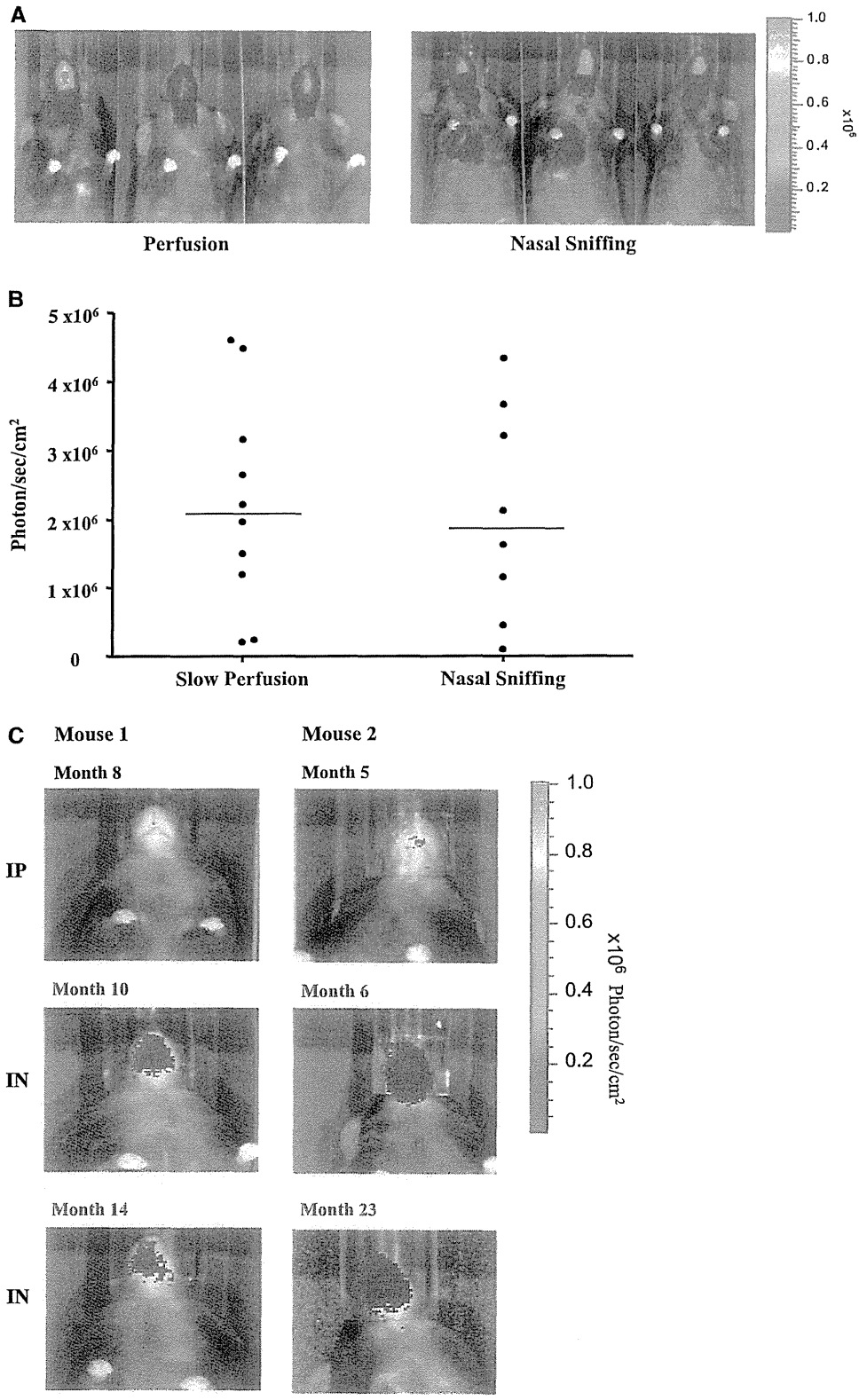


Figure 2. F/HN simian immunodeficiency virus (SIV) requires only short contact time to achieve efficient transduction. Luciferase (Lux) expression was compared in mice receiving F/HN-SIV-Lux ($3–4 \times 10^8$ TU/mouse in 100 μl) by slow perfusion (1.3 μl/min, n = 10) or as bolus administration by nasal sniffing (n = 8). (*A*) Bioluminescence *in vivo* signal in the mouse nose. Representative images of three mice are shown. (*B*) Quantification of *in vivo* bioluminescence in mouse nose after slow perfusion or nasal sniffing of the vector. Each *dot* represents one animal. *Horizontal bars* represent the group median. (*C*) Bioluminescence *in vivo* signal in mouse nose after intraperitoneal (IP) or intranasal (IN) administration of luciferin. Representative images of two of five mice are shown. Lux expression after slow perfusion of the F/HN-SIV-Lux has been published previously (11).

administration is feasible and significantly increases transduction efficiency.

Monthly Repeat Administration to the Lung Is Feasible

Although a single dose of F/HN-SIV generates persistent gene expression for approximately 2 years in mice (lifetime of the animal) a single dose is unlikely sufficient to achieve clinical benefit for the lifetime of a patient with CF. Therefore, a crucial question is whether the vector can produce gene expression on repeated administration to the lung. As shown previously, daily administration was feasible, but this timeframe is unlikely to be sufficient for the development of effective immune responses to the viral vector.

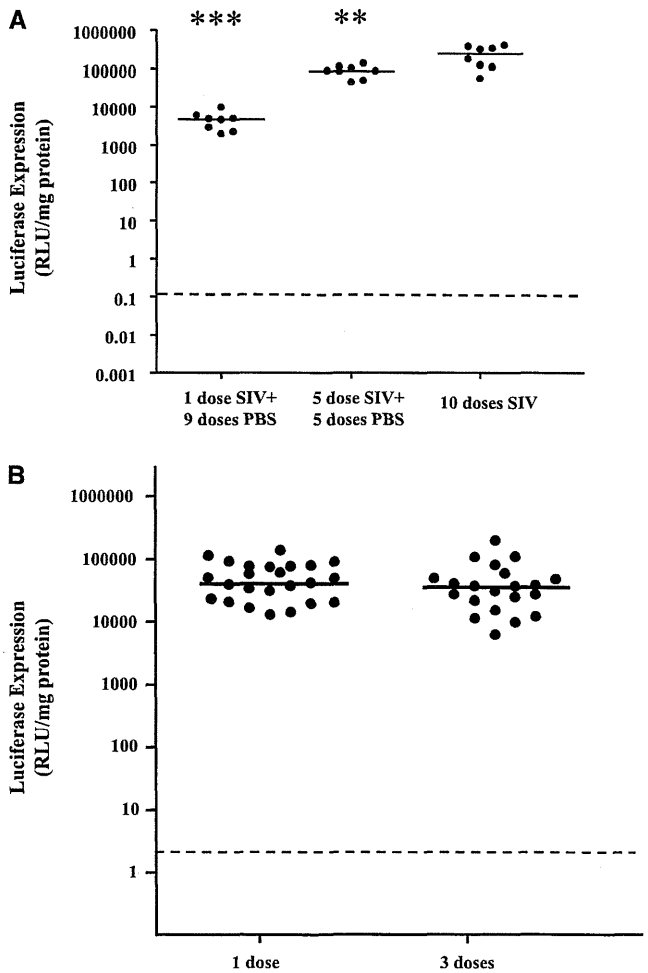


Figure 3. Daily and monthly repeat administration to the lung is feasible. (*A*) Daily readministration. Mice (n = 8 per group) were treated with either F/HN simian immunodeficiency virus (SIV) luciferase (Lux) for 10 days (10^6 TU/day) or with D phosphate buffered saline (PBS) for 5 days followed by 5 days of F/HN-SIV-Lux (10^6 TU/day) or with D-PBS for 9 days followed by 1 day of F/HN-SIV-Lux (10^6 TU). Lux expression was analyzed 28 days after F/HN-SIV-Lux administration. (*B*) Monthly readministration. Mice were either treated with one dose of F/HN-SIV-Lux (single-dose group) or two doses of F/HN-SIV-GFP (Day 0, Day 28), followed by F/HN-SIV-Lux on Day 56 (repeat-dose group). All mice receive 10^7 TU/dose (n = 20 per group) and gene expression was analyzed 28 days after F/HN-SIV-Lux administration. Each *dot* represents one animal. *Horizontal bars* represent the group median. *Dotted line* represents negative control values, ***$P < 0.0001$ when compared with 5 and 10 doses, **$P < 0.01$ when compared with 10 doses. RLU = relative light units.

We therefore conducted repeat administration experiments at monthly dosing intervals, a period we have shown to be sufficient for the development of an immune response to other viral vectors (5, 17). Mice were either treated with one dose of F/HN-SIV-Lux (single-dose group) or two doses of F/HN-SIV-GFP (Day 0, Day 28), followed by F/HN-SIV-Lux on Day 56 (repeat-dose group). All mice received 10^7 TU in 100 μl per dose (n = 20 per group) and gene expression was analyzed 28 days after F/HN-SIV-Lux administration. All mice received only one dose of vector carrying a luciferase reporter gene to avoid interference of antiluciferase antibodies. As shown in Figure 3B luciferase expression levels after one and three doses of the viral vector were identical and at this vector titer 4 log orders above background levels ($P < 0.01$).

No Evidence of Chronic Toxicity Was Seen during a Two-Year Follow-up Period

Chronic toxicity caused by insertional mutagenesis is a potential concern with this vector. Here, we compared 24-months survival of mice treated with F/HN-SIV-Lux (10^7 TU/mouse, n = 99 at start of the experiment) or D-PBS (n = 48 at start of the experiment). Mortality (Figure 4A) and weight (Table 1) in both groups was similar. We also assessed the lungs of D-PBS– or F/HN-SIV-Lux– (10^7 TU/mouse, n = 12 per group) treated mice histologically 24 months after nasal sniffing; there were no differences in any of the key histologic markers (Figures 4B–4D). Eleven D-PBS and seven F/HN-SIV–treated mice were culled because they showed signs of illness or were found dead during the study. Histologic examination revealed a range of pathology commonly found in aging C57BL/6N mice equally distributed between the two groups (Table 2).

F/HN-SIV Transduction of Human *Ex Vivo* Models Is Feasible

F/HN-SIV achieves persistent gene expression in differentiated human ALI cultures. To assess the efficiency of the viral vector in relevant human tissues we transduced human ALIs with F/HN-SIV-Lux at multiplicity of infection (MOI) 25 or 250 (n = 5 per group), with D-PBS control (n = 3), or with GL67A, the most efficient nonviral gene transfer agent for airway epithelium. The latter was complexed with a eukaryotic expression plasmid expressing luciferase (10 μg pCIKLux per ALI equivalent to 3×10^{12} plasmids and an approximate MOI of 7×10^6) (n = 3) and luciferase expression followed using BLI for 3 months (representative images are shown in Figure 5A). F/HN-SIV–mediated expression was dose-related and persisted for at least 3 months without the loss of activity. In contrast, transfection with GL67A/pCIKLux was only barely detectable at an early time-point (Day 2) after transfection (Figure 5B). Lipid-mediated gene transfer was associated with cell damage as indicated by the appearance of small holes in the ALIs; this was not seen with exposure of the ALIs to the lentiviral vector (data not shown).

F/HN-SIV transduces primary human pulmonary cells. We assessed if F/HN-SIV transduces primary human airway epithelial cells obtained from nasal brushings or human lung slices generated from lung resection tissue (Figure 6A). Nasal brushings were transduced with F/HN-SIV-Lux at an MOI of 25 and 250 or treated with D-PBS (n = 6 per group) and luciferase expression was quantified 24 hours after transduction (cell viability precludes longer time scales). Luciferase expression was significantly ($P < 0.005$) increased compared with PBS control subjects (Figure 6B). These results were confirmed in an independent experiment (MOI 25, $9.7 \times 10^6 \pm 0.9 \times 10^6$; MOI 250, $1.5 \times 10^7 \pm 1.6 \times 10^6$; negative control subjects, 2,762 ± 241 RLU/mg protein, n = 4 per group)

F/HN-SIV achieves persistent gene expression in human and sheep lung slices. Precision-cut human lung slices were transduced with F/HN-SIV-Lux (2×10^7 TU/slice), or remained untransduced, and luciferase expression quantified for 14 days after transduction. Assessment of later time-points is currently not feasible because of limited tissue viability (Figure 6C). Two independent experiments were performed (*see open* and *closed* symbols in the figure) but data were pooled to allow for robust statistical analysis. Significant ($P < 0.001$) and stable gene expression was detectable for at least 14 days ($P < 0.05$) after gene transfer.

We also repeated these experiments in sheep lung slices (two independent experiments), which generally survive longer than human lung slices when cultured (unpublished data). Significant ($P < 0.001$ to $P < 0.05$) and stable luciferase expression was detectable at all time-points for at least 26 days after transduction

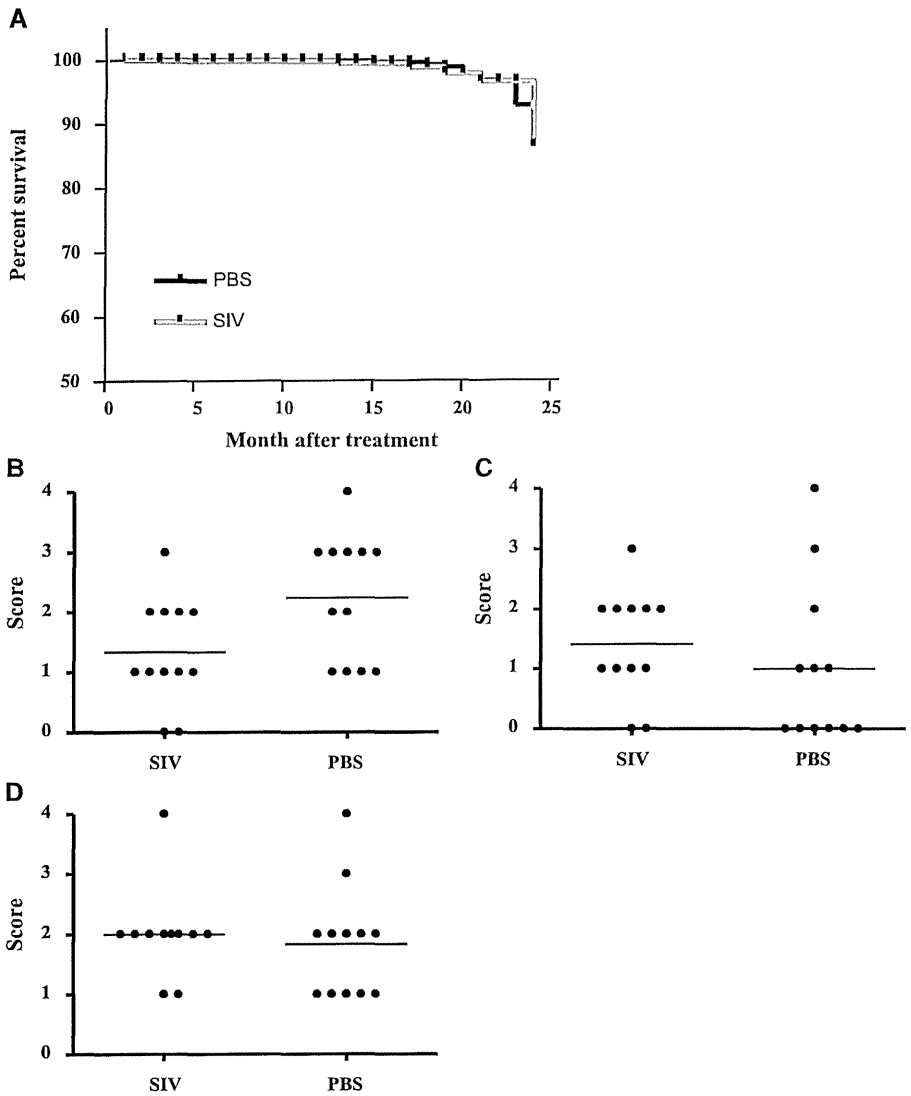


Figure 4. No evidence of chronic toxicity during 2-year follow-up period. (*A*) Twenty-four months survival of mice treated with F/HN simian immunodeficiency virus (SIV) luciferase (Lux) (10^7 TU/mouse, n = 99 at start of the experiment) or phosphate-buffered saline (PBS) (n = 48 at start of the experiment) by nasal sniffing. Comparison of lymphocytes (*B*), edema (*C*), and inflammation (*D*) in mouse lung 24 months after F/HN-SIV-Lux or PBS treatment. Each *dot* represents one animal. *Horizontal bars* represent the group median.

(Figure 6D). These results indicate that F/HN–SIV is able to transduce primary human and sheep pulmonary cells and provides persistent expression.

DISCUSSION

The efforts of one of our team to improve pulmonary gene transfer and enable gene therapy for the treatment of lung diseases, such as CF, have led to the development of a lentiviral vector pseudotyped with the SeV envelope proteins F and HN (12). Moving novel therapies to the clinic requires that relevant evidence for safety and efficacy is gathered in appropriate models. Here, we begin to place this vector onto a translational pathway to the clinic and provide a body of supportive evidence for F/HN-pseudotyped SIV as a potential gene transfer agent for CF. We show that (*1*) a single dose produces lung expression for the life-time of the mouse (~2 yr); (*2*) only brief contact time (seconds) is needed to achieve transduction; (*3*) repeated daily administration leads to a dose-related increase in gene expression; (*4*) repeated monthly administration to mouse lower airways is feasible without loss of gene expression; (*5*) there is no evidence of chronic toxicity during a 2-year study period; and (*6*) F/HN-SIV transduction generates persistent gene expression in human differentiated airway cultures and freshly obtained human lung slices and transduces freshly obtained primary human airway epithelial cells.

At least two previous studies have shown that lentiviral vector–mediated transduction of mouse lung leads to persistent (15 and 24 mo) gene expression in the murine lower airways (18, 19). However, both studies used a VSV-G–pseudotyped lentiviral vector, which almost exclusively transduces alveolar macrophages, rather than lung epithelial cells. In addition to the airway epithelium, the target for CF gene therapy, cells in the peripheral lung (a mixture of pneumocytes and macrophages) are transduced after bolus administration of the viral vector by nasal sniffing, which may lead to pooling of liquid in the

TABLE 1. MOUSE WEIGHTS *6*, *15*, AND *24* MONTHS AFTER LUNG TRANSDUCTION WITH F/HN-SIV-LUX OR PBS ADMINISTRATION

Months After Treatment	PBS-treated Mice 8 g	SIV-treated Mice 8 g
6	24.9 (0.5) n = 48	25.4 (0.3) n = 71
15	30.7 (0.7) n = 47	31.4 (0.8) n = 35
24	30.1 (1.4) n = 36	29.1 (1.1) n = 12

Definition of abbreviations: LUX = luciferase; PBS = phosphate-buffered saline; SIV = simian immunodeficiency virus.

Mean weights (± SEM) are shown. There were no differences between PBS- and SIV-treated mice.

TABLE 2. CAUSE OF MORTALITY OF PBS- AND F/HN-SIV–TREATED MICE OVER A 24-MONTH PERIOD

Treatment	Death (*mo after treatment*)	Diagnosis
PBS	15	Found dead
PBS	18	Lymphoma
PBS	19	Lymphoma
PBS	20	Lymphoma
PBS	20	Inconclusive
PBS	21	No pathology found
PBS	21	Lymphoma
PBS	23	No pathology found
PBS	23	Found dead
PBS	23	Lymphoma
PBS	24	Found dead
SIV	3	Peritonitis
SIV	13	Found dead
SIV	17	Found dead
SIV	19	Pancreatic tumor
SIV	21	Multiorgan inflammation
SIV	24	Found dead
SIV	24	Multifocal lymphocytic inflammation

Definition of abbreviations: PBS = phosphate-buffered saline; SIV = simian immunodeficiency virus.

peripheral lung. To determine transduction efficiency of airway epithelium more accurately the viral vector needs to be administered by nebulization. Further work is now warranted to determine whether F/HN-SIV is stable in clinically approved nebulizers and that virus production can be scaled up to the extent required for clinical studies. We are currently assessing if F/HN-SIV is stable in clinically approved nebulizers and preliminary results indicate that infectivity after nebulization is retained (data not shown); and scaling up vector production to allow us to move into *in vivo* nebulization experiments in the near future.

Although expression levels in the lung are completely stable over the 2-year study period, those in the nasal epithelium declined by approximately 60%. The latter is consistent with our previous data (11) and may relate to different cell types being transduced in the lung (airway epithelium and alveolar cells) and nose (mainly ciliated airway epithelial cells) (11) and different turnover rates of these cells at these two sites. Although, we do not have information about turnover of nasal airway epithelium, Rawlins and Hogan (13) have shown that the half-life of ciliated airway epithelial cells in the mouse trachea and lung differs (trachea, average half-life 6 mo; lung, average half-life 17 mo).

We have previously shown that prolongation of contact time between nonviral gene transfer agents and the airway epithelium significantly increases gene expression (20). In our previous study, we therefore slowly perfused F/HN-SIV (1.3 μl/min) onto the mouse nasal epithelium to maximize transduction (11). Transduction efficiency in the nasal epithelium after administration of a bolus of fluid that is rapidly (seconds) sniffed into the lung led to similar levels of photon emission. This implies that brief contact time between F/HN-SIV and the target cell may be sufficient for efficient vector uptake into the cells, which is an important factor for clinical translation. F/HN-SIV therefore mimics SeV (2) from which the F and HN envelope proteins were derived.

We and others have previously shown that repeated administration of lentiviral vectors to the mouse nasal epithelium is feasible (10, 11). To move our translational research a step closer to clinical relevance we now assessed repeat administration of the vector in the lung. Two types of repeat administration experiments were performed: daily and monthly. The former was assessed because it is conceivable that the total volume required for delivery of a specific vector titer to the human lung may be too large for a single dose and administration may have to be

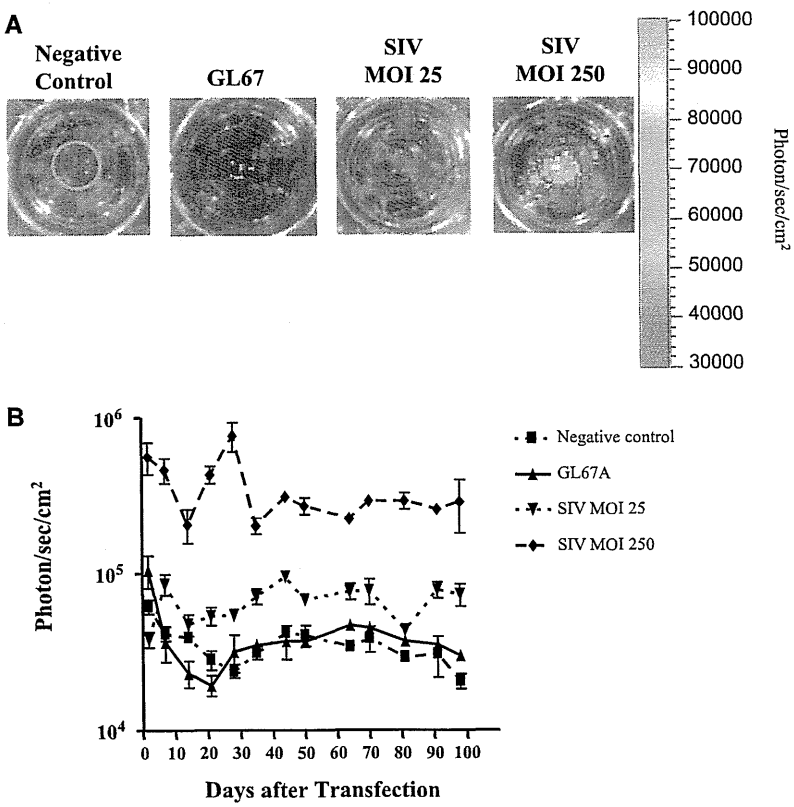


Figure 5. Persistent gene expression in differentiated air–liquid interface (ALI) cultures. Differentiated human ALI cultures were transduced with F/HN simian immunodeficiency virus (SIV) luciferase (Lux) at multiplicity of infection (MOI) 25 or 250 (n = 5 per group), transfected with GL67A complexed to a eukaryotic expression plasmid expressing Lux (10 μg pCIKLux per ALI equivalent to 3×10^{12} plasmids and an approximate MOI of 7×10^6) (n = 3) or treated with phosphate-buffered saline (PBS) (negative control, n = 3). Lux expression was followed using bioluminescence imaging for 3 months. (*A*) Representative images are shown. (*B*) Quantification of Lux expression over 3 months.

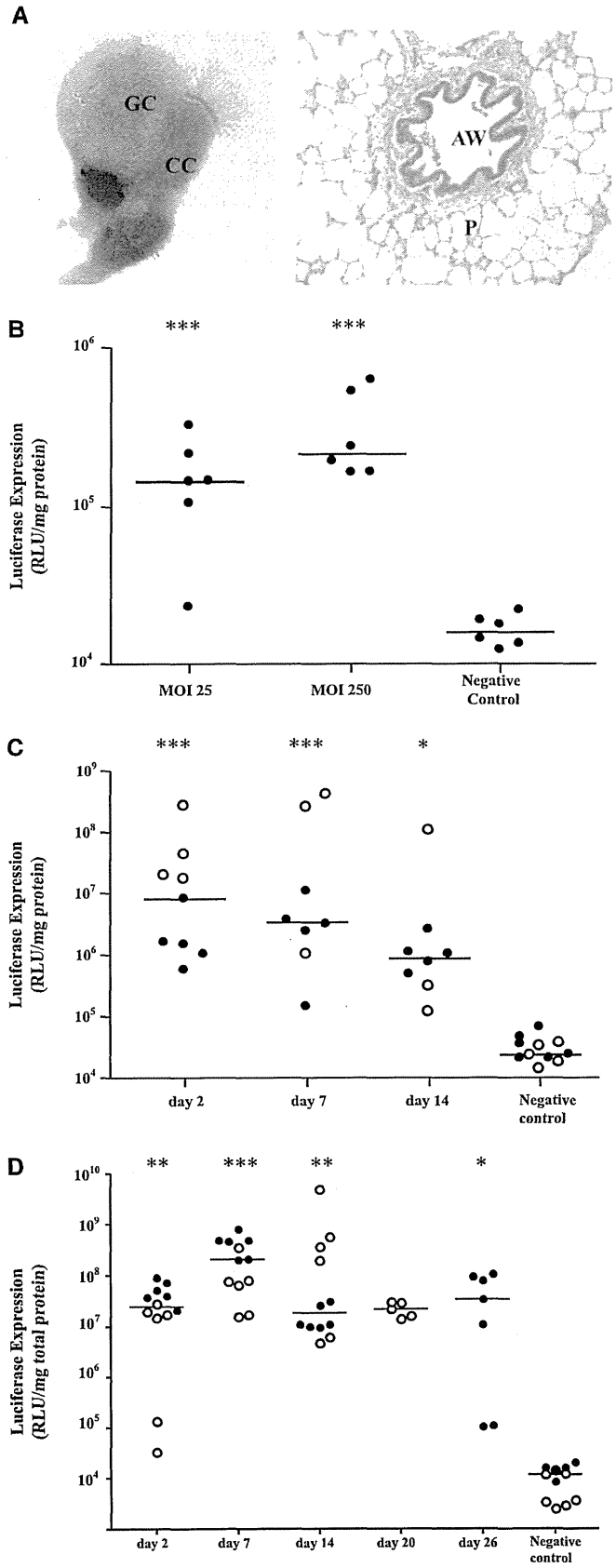


Figure 6. F/HN simian immunodeficiency virus (SIV) transduces primary human pulmonary cells. (*A*) Microscopic section showing primary human airway epithelial cells obtained from nasal brushings (*left*, GC = goblet cell, CC = ciliated airway epithelial cell) or human lung slices (*right*, AW = airway, P = parenchyma). (*B*) Nasal brushings were transduced with F/HN-SIV-luciferase (Lux) at a multiplicity of infection (MOI) of 25 and 250 or treated with phosphate-buffered saline (PBS) (negative control) and Lux expression was quantified 24 hours after transduction. Each *dot* represents one sample. *Horizontal bars* represent the group median. ***$P < 0.005$ when compared with negative controls. (*C*) Human lung slices were transfected with F/HN-SIV-Lux (2×10^7 TU/slice) or remained untransfected (negative control) and Lux expression quantified 2, 7, and 14 days after transduction. Two independent experiments were performed (*open* and *closed* symbols). (*D*) Sheep lung slices were transfected with F/HN-SIV-Lux (2×10^7 TU/slice) or remained untransfected (negative control) and Lux expression quantified up to 26 days after transduction. Two independent experiments were performed (*open* and *closed* symbols). Each *dot* represents one animal. *Horizontal bars* represent the group median. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ when compared with negative control. RLU = relative light units.

split into several doses to accommodate the volume. Here, we show that daily repeat administration is feasible and leads to a dose-related increase in gene expression. Although important, we have not yet addressed the question if "split-dose" delivery of a specific vector titer (e.g., 10 doses of 10^7 TU) offers advantages over "single-dose" delivery of the same vector titer (e.g., one dose of 10^8 TU), which may also be a relevant translational research question. Although efficient daily-repeat administration is encouraging, this time-frame is generally not considered to be sufficient to induce robust immune responses to the viral vector. To confirm that long-term F/HN-SIV repeat administration to the lower airways is feasible viral vector was administered monthly over a 3-month period. This dosing interval may be of clinical relevance and is also an accepted timeframe for the induction of robust immune responses. Uniquely for a viral vector (in our hands), luciferase expression levels after one and three doses were identical and more than 4 log orders higher than in untransduced control mice.

Another important consideration for translational development of a gene transfer agent is safety. The occurrence of leukemia and myelodysplasia in some patients with primary immunodeficiencies after treatment with γ retroviral vector-transduced bone marrow (21) raises concerns about the safety of integrating viral vectors. The risk of insertional mutagenesis in slowly turning over differentiated lung epithelium is unknown, but is likely to be lower than in rapidly dividing bone marrow cells. In addition, the improved design of self-inactivating lentiviral vectors has improved safety (21). Here we compared survival, weight, and lung histology during a 24-month study period and did not see any differences between F/HN-SIV– and PBS-treated mice. We included only female mice in the study because they can be more readily housed together for prolonged periods of time. It is unlikely that the toxicology profile in male mice would be significantly different. However, before progression into clinical trial a good laboratory practice (GLP)-toxicology study, including male and female animals and dose-ranging, has to be performed. Despite the encouraging toxicology profile of our current vector configuration we plan to assess further modifications before final vector selection for clinical trials, including the use of mammalian promoters and ubiquitous chromatin opening elements. The former have been shown to reduce the risk of insertional mutagenesis compared with strong viral promoters (22), whereas the latter may reduce gene silencing (22). The ability to administer

this vector repeatedly and efficiently also opens the possibility of using nonintegrating lentiviral vectors (23), which may further reduce the genotoxic risk.

As part of our translational pathway we next determined whether F/HN-SIV is able to transduce human tissues. We have previously shown that F/HN-SIV can transduce fully differentiated human ALI cultures when analyzed 5 days after transduction (11). These cultures mimic human airway epithelium and are difficult to transfect (24). Here, we have extended these studies and show that F/HN-SIV–mediated expression in ALIs is stable; persists for at least 3 months (the length of the experiment); and is higher than GL67A-mediated expression, which in our hands is currently the most efficient nonviral vector. Preliminary experiments indicated that in contrast to lipid-mediated gene transfer, F/HN-SIV does not seem to damage the ALI cultures, but more extensive studies are required to determine acute toxicity *in vitro* and *in vivo*.

To further evaluate the viral vector in even more relevant tissues we assessed freshly obtained human primary airway cells and showed that F/HN-SIV-Lux transduction leads to significant levels of luciferase expression. We performed only short-term (24 h) experiments to avoid potential cell division, which would have affected interpretation of the results. Collection of primary cells by nasal brushings exposes the basolateral membrane of these cells and we cannot exclude the possibility that the vector may have entered the cells by this route. However, experiments performed in mice *in vivo* and in ALI cultures clearly show that F/HN-SIV can transduce airway epithelial cells when applied to the apical membrane without the need to coadminister agents to open tight junctions. There was no difference in gene expression when cells were transduced with an MOI of 25 or 250. In future experiments the MOI will be further reduced to determine the minimum MOI for efficient transduction.

We also assessed F/HN-SIV in precision-cut human lung slices. Expression in both experiments was stable and persisted for at least 14 days, after which the experiment was terminated because of reduced tissue viability. Although we have not determined which cells express the recombinant protein these studies show that persistent SIV-mediated transduction into human pulmonary cells is feasible and is not prevented by viral restriction factors. We also transduced ovine lung slices, which are generally more resilient when cultured (Nikki Newman, personal communication); stable luciferase expression was detectable for at least 26 days. These data support the future use of these models in the clinical development of this vector.

SeV shares high sequence homology with human parainfluenza virus and preexisting immunity may be a factor. Interestingly, Slobod and coworkers (25) assessed SeV-vaccination against human parainfluenza virus and reported evidence of immunogenicity in three of nine vaccinees despite preexisting, cross-reactive immunity presumably induced by previous exposure to human parainfluenza virus-1. Although this initial study is encouraging, the effects of preexisting humoral immunity on virus transduction efficiency is something we will address as a high priority.

Gene transfer to the airway epithelium is more difficult than originally anticipated. Until now viral gene transfer agents have not been useful for the treatment of chronic lung disease, such as CF, because of immunogenicity, which prevents successful repeat administration. Lentivirus-based vectors are a notable exception. Moving novel therapies to the clinic requires that relevant evidence for safety and efficacy is gathered in appropriate models. Here, we begin to place this vector onto a translational pathway to the clinic and provide a body of supportive evidence for F/HN-pseudotyped SIV as a potential gene transfer agent for CF including (*1*) lifetime gene expression and efficient repeat administration in mouse lung, (*2*) lack of chronic toxicity, and (*3*) persistent gene expression in human *ex vivo* models.

The data presented here support F/HN-SIV as a promising vector for pulmonary gene therapy, with potential for many diseases including CF. We are currently undertaking the next critical steps (vector optimization and virus production) in this developmental process toward clinical evaluation and are improving vector optimization and viral production methods to support progression into early phase trials.

Author disclosures are available with the text of this article at www.atsjournals.org.

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References

1. Griesenbach U, Alton EW. Progress in gene and cell therapy for cystic fibrosis lung disease. *Curr Pharm Des* 2012;18:642–662.
2. Yonemitsu Y, Kitson C, Ferrari S, Farley R, Griesenbach U, Judd D, Steel R, Scheid P, Zhu J, Jeffery PK, *et al.* Efficient gene transfer to airway epithelium using recombinant Sendai virus. *Nat Biotechnol* 2000;18:970–973.
3. Griesenbach U, Cassady RL, Ferrari S, Fukumura M, Muller C, Schmitt E, Zhu J, Jeffery PK, Nagai Y, Geddes DM, *et al.* The nasal epithelium as a factory for systemic protein delivery. *Mol Ther* 2002;5:98–103.
4. Harvey BG, Leopold PL, Hackett NR, Grasso TM, Williams PM, Tucker AL, Kaner RJ, Ferris B, Gonda I, Sweeney TD, *et al.* Airway epithelial CFTR mRNA expression in cystic fibrosis patients after repetitive administration of a recombinant adenovirus. *J Clin Invest* 1999;104:1245–1255.
5. Sumner-Jones SG, Davies LA, Varathalingam A, Gill DR, Hyde SC. Long-term persistence of gene expression from adeno-associated virus serotype 5 in the mouse airways. *Gene Ther* 2006;13:1703–1713.
6. Limberis M, Anson DS, Fuller M, Parsons DW. Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer. *Hum Gene Ther* 2002;13:1961–1970.
7. Cmielewski P, Anson DS, Parsons DW. Lysophosphatidylcholine as an adjuvant for lentiviral vector mediated gene transfer to airway epithelium: effect of ACYL chain length. *Respir Res* 2010;11:84.
8. Medina MF, Kobinger GP, Rux J, Gasmi M, Looney DJ, Bates P, Wilson JM. Lentiviral vectors pseudotyped with minimal filovirus envelopes increased gene transfer in murine lung. *Mol Ther* 2003;8:777–789.
9. McKay T, Patel M, Pickles RJ, Johnson LG, Olsen JC. Influenza M2 envelope protein augments avian influenza hemagglutinin pseudotyping of lentiviral vectors. *Gene Ther* 2006;13:715–724.
10. Sinn PL, Arias AC, Brogden KA, McCray PB Jr. Lentivirus vector can be readministered to nasal epithelia without blocking immune responses. *J Virol* 2008;82:10684–10692.
11. Mitomo K, Griesenbach U, Inoue M, Somerton L, Meng C, Akiba E, Tabata T, Ueda Y, Frankel GM, Farley R, *et al.* Toward gene therapy for cystic fibrosis using a lentivirus pseudotyped with Sendai virus envelopes. *Mol Ther* 2010;18:1173–1182.
12. Kobayashi M, Iida A, Ueda Y, Hasegawa M. Pseudotyped lentivirus vectors derived from simian immunodeficiency virus SIVagm with envelope glycoproteins from paramyxovirus. *J Virol* 2003;77:2607–2614.
13. Rawlins EL, Hogan BL. Ciliated epithelial cell lifespan in the mouse trachea and lung. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L231–L234.
14. Ohmori K, Takeda S, Miyoshi S, Minami M, Nakane S, Ohta M, Sawa Y, Matsuda H. Application of HVJ-liposome mediated gene transfer in lung transplantation-distribution and transfection efficiency in the lung. *Eur J Cardiothorac Surg* 2005;27:768–773.
15. Xenariou S, Griesenbach U, Liang HD, Zhu J, Farley R, Somerton L, Singh C, Jeffery PK, Ferrari S, Scheule RK, *et al.* Use of ultrasound to enhance nonviral lung gene transfer in vivo. *Gene Ther* 2007;14:768–774.
16. Griesenbach U, Meng C, Farley R, Gardner A, Brake MA, Frankel GM, Gruenert DC, Cheng SH, Scheule RK, Alton EW. The role of doxorubicin in non-viral gene transfer in the lung. *Biomaterials* 2009; 30:1971–1977.
17. Griesenbach U, Boyton RJ, Somerton L, Garcia SE, Ferrari S, Owaki T, Ya-Fen Z, Geddes DM, Hasegawa M, Altmann DM, *et al.* Effect of tolerance induction to immunodominant T-cell epitopes of Sendai

virus on gene expression following repeat administration to lung. *Gene Ther* 2006;13:449–456.
18. Hirayama S, Sato M, Liu M, Loisel-Meyer S, Yeung JC, Wagnetz D, Cypel M, Zehong G, Medin JA, Keshavjee S. Local long-term expression of lentivirally delivered IL-10 in the lung attenuates obliteration of intrapulmonary allograft airways. *Hum Gene Ther* 2011;22: 1453–1460.
19. Wilson AA, Murphy GJ, Hamakawa H, Kwok LW, Srinivasan S, Hovav AH, Mulligan RC, Amar S, Suki B, Kotton DN. Amelioration of emphysema in mice through lentiviral transduction of long-lived pulmonary alveolar macrophages. *J Clin Invest* 2010;120:379–389.
20. Griesenbach U, Meng C, Farley R, Wasowicz MY, Munkonge FM, Chan M, Stoneham C, Sumner-Jones SG, Pringle IA, Gill DR, *et al.* The use of carboxymethylcellulose gel to increase non-viral gene transfer in mouse airways. *Biomaterials* 2010;31:2665–2672.
21. Booth C, Gaspar HB, Thrasher AJ. Gene therapy for primary immunodeficiency. *Curr Opin Pediatr* 2011;23:659–666.
22. Mukherjee S, Thrasher AJ. Progress and prospects: advancements in retroviral vector design, generation, and application. *Hum Gene Ther* 2011;22:1171–1174.
23. Banasik MB, McCray PB Jr. Integrase-defective lentiviral vectors: progress and applications. *Gene Ther* 2010;17:150–157.
24. Griesenbach U, Vicente CC, Roberts MJ, Meng C, Soussi S, Xenariou S, Tennant P, Baker A, Baker E, Gordon C, *et al.* Secreted Gaussia luciferase as a sensitive reporter gene for in vivo and ex vivo studies of airway gene transfer. *Biomaterials* 2011;32:2614–2624.
25. Slobod KS, Shenep JL, Lujan-Zilbermann J, Allison K, Brown B, Scroggs RA, Portner A, Coleclough C, Hurwitz JL. Safety and immunogenicity of intranasal murine parainfluenza virus type 1 (Sendai virus) in healthy human adults. *Vaccine* 2004;22:3182–3186.

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ORIGINAL ARTICLE *Genetics*

Identification and characterization of an adenine to guanine transition within intron 10 of the factor VIII gene as a causative mutation in a patient with mild haemophilia A

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Summary. Haemophilia A is caused by various genetic mutations in the factor VIII gene (*F8*). However, after conventional analysis, no candidate mutation could be identified in the *F8* of about 2% of haemophilia A patients. The *F8* of a patient with mild congenital haemophilia A, in whom no candidate mutation was found in the exons or their flanking regions, was analysed in detail to identify the patient's aetiological genetic abnormality. We also characterized anti-FVIII antibody (inhibitor) development in this patient. Genomic DNA analysis revealed an adenine to guanine transition deep inside intron 10 (c.1478 + 325A>G) of *F8* as a causative mutation. Analysis of the transcripts demonstrated that the majority of the patient's transcript was abnormal, with 226 bp of the intronic sequence inserted between exon 10 and 11. However, the analysis also indicated the existence of a small amount of normal transcript. Semi-quantification of ectopic *F8* mRNA showed that about one-tenth of the normal mRNA level was present in the patient. After the use of a recombinant FVIII concentrate, the presence of an inhibitor was confirmed. The inhibitor was characterized as oligoclonal immunoglobulin IgG4 directed against both the A2 domain and light chain of the FVIII molecule with type I reaction kinetics of inhibition of FVIII activity. When no mutations are found by conventional analysis, deep intronic nucleotide substitutions may be responsible for mild haemophilia. The inhibitor development mechanism of the patient producing some normal FVIII was thought to be of interest.

Keywords: factor VIII, haemophilia A, inhibitor, intron, mutation

Introduction

Haemophilia A (MIM + 306700) is an X-linked bleeding disorder caused by a genetic defect in the coagulation factor VIII gene (*F8*). The *F8* is located on the most distal band of chromosome X (Xq28) and spans 186 Kb [1]. This large gene consists of 26 exons encoding 2351 amino acids [2]. Since the cloning of *F8* in 1984, there has been a robust effort to identify the mutation within *F8* responsible for haemophilia. Nowadays, more than 900 unique mutations have been identified and registered in a worldwide mutation database, HADB (http://hadb.org.uk, also known as HAMSTeRS, The Haemophilia A Mutation, Structure, Test and Resource Site). Various types of genetic mutation which cause haemophilia A have been detected in *F8*. However, in approximately 2% of haemophilia A patients, no genetic mutation can be found in *F8*, even after nucleotide sequencing including the 5′-untranslated region, the entire coding region, exon/intron boundaries and the 3′-untranslated region [3,4]. In these cases, the possibility that some causative mutations might be located in a further unanalysed region of *F8* is still suspected. For example, although it occupies a large part of the gene, it is difficult to examine deep inside intron in detail, which leaves this relatively unanalysed region as a strong candidate for undetected mutations.

The most serious complication of factor VIII (FVIII) replacement therapy in haemophilia A is the development of alloantibodies against transfused FVIII. This markedly attenuates the effectiveness of FVIII replacement therapy. In general, the incidence of inhibitor

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development in patients with haemophilia A is estimated to be 20–30% [5–7]. Severe patients who carry null mutations (e.g. intron 22 inversion, large deletion and nonsense mutation) are usually at high risk of inhibitor development [8,9], because their mutations do not produce endogenous FVIII protein. Conversely, mild/moderate patients who carry less severe genetic defects (e.g. missense mutation) are usually at low risk of inhibitor development, because they can produce some endogenous FVIII protein. However, the endogenous FVIII molecule is an abnormal FVIII mutant and is recognized as "self" in the patients. Therefore, inhibitor development in mild/moderate patient is observed when a transfused normal FVIII is recognized as "non-self". The incidence of inhibitor development is influenced not only by the genetic abnormality of the patient but also by hereditary background and environmental factors [9].

In this article, we describe a patient with mild haemophilia A who developed a high titre inhibitor. Our genetic analysis revealed that the patient carried a novel adenine to guanine transition deep inside intron 10 of the *F8* as a candidate causative mutation. Furthermore, mRNA analysis revealed that a FVIII protein produced by the patient might be normal. The development of inhibitor in this inherently mild patient is of interest.

Materials and methods

Patient

A 71-year-old man with a history of stomach cancer was diagnosed as suffering from mild haemophilia A (FVIII activity 10%) before a surgical operation at the age of 60. Although, he described some indications of haemostasis difficulty, for example in tooth extractions etc. during childhood, the patient had no history of haemorrhage that required treatment. There were no cases of haemophilia amongst the patient's relatives. Three months after the first infusion of recombinant FVIII (Kogenate; Bayer), about 20 exposure days, anti-FVIII antibody was detected for the first time. The study was approved by the Ethics Committee of Tokyo Medical University and written informed consent was obtained from the patient. The studies were carried out in accordance with the principles of the Declaration of Helsinki.

Genomic DNA and total RNA preparation

Genomic DNA was extracted from peripheral blood cells using the EZ1 DNA Blood 350 μL Kit (Qiagen, Hilden, Germany) on a BioRobot EZ1 workstation (Qiagen). Total RNA was isolated from peripheral blood cells using a QIAamp® RNA Blood Mini Kit (Qiagen) or PAXgene® Blood RNA Kit (Qiagen). Both preparations were performed following the manufacturer's instructions.

F8 amplification and sequencing

The *F8* entire coding regions, exon/intron boundaries, and the 5′ and 3′-untranslated region, were amplified by PCR with 36 sets of primers. We designed most of the PCR primers used in this study, although some were as described previously [4]. The M13 consensus sequence was added to the 5′ end of all primers for direct sequencing. The amplified PCR products were electrophoresed on a 3% agarose gel and were extracted using QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were directly sequenced using the M13 consensus sequence as primer. Cycle sequencing was performed using the BigDye Terminator kit. Unincorporated BigDye Terminators and unused primers were removed using the Big Dye XTerminator Purification Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Sequencing was performed on a 48-capillary 3730 DNA Analyzer (Applied Biosystems).

Factor VIII gene mRNA analysis

To analyse the splicing of *F8*, nested amplification for *F8* cDNA was performed using a Qiagen OneStep RT-PCR Kit (Qiagen) and with primers that were reported by El-Maarri *et al.* [10]. Ectopic *F8* mRNA level was relatively quantified by a real-time PCR technique. Briefly, reverse transcription was performed using a commercially available kit (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems) according to the manufacturer's instructions. The *F8* cDNA was then amplified and analysed by commercially available TaqMan gene expression assays (Hs00240767_m1, Hs01109547_m1; Applied Biosystems). Relative quantification of *F8* mRNA expression was performed using the comparative Ct method. The *F8* expression level was normalized with endogenous control β-actin (Hs99999903_m1, Applied Biosystems). Total RNA from caucasian male liver (FirstChoice® Total RNA) was used as a reference. All amplifications were carried out at least in triplicate.

Coagulation test

We measured FVIII activity (FVIII:C) level using a one-stage clotting assay with the use of commercial aPTT reagents (HemosILTM APTT-SP reagent; Instrumentation Laboratory), FVIII-deficient plasma (George King Bio-Medical, Overland Park, KS, USA) and an ACL-9000 automatic coagulation analyser (Instrumentation Laboratory, Bedford, MA, USA). Anti-FVIII antibody (inhibitor) level was measured by the Bethesda method [11].

Characterization of IgG subclass

Recombinant factor VIII (rFVIII) was used as an antigen and coated onto microtitre plate wells. The patient's plasma was reacted with coated rFVIII and subsequently IgG subclass was detected using a Human IgG Subclass Screening Kit (Cygnus Technologies, Southport, NC, USA).

Western blotting analysis

Polyacrylamide gel electrophoresis with sodium dodecyl sulphate was performed with polyacrylamide gradient gels (2%–15%) (Multigel 2/15; Daiichi Pure Chemicals, Tokyo, Japan). The rFVIII non-treated, or treated with α-thrombin, was loaded onto gels under non-reducing conditions and then transferred onto PVDF membrane. The IgG in the patient's plasma was reacted with rFVIII and subsequently detected by HRP labelled anti-human IgG (Biosource, Camarillo, CA, USA). Immunoreactions were visualized with a Konica Immunostain HRP-1000 kit (Konica Corporation, Tokyo, Japan).

Results

Identification of candidate mutation

Nucleotide sequencing of entire coding regions, exon/intron boundaries and the 5′ and 3′-untranslated region of *F8* was performed. However, no genetic abnormality recognized as causative of haemophilia A was detected. The only nucleotide substitution, an adenine to guanine transition, was unexpectedly detected 325 bp downstream from the 3′ end of exon 10 (c.1478 + 325A>G) (Fig. 1). This transition was not found in *F8* of 103 Japanese males and has not been registered in any database as a mutation or a polymorphism. These results strongly suggest that the transition is very rare and may be involved in FVIII deficiency in this patient.

mRNA analysis

Analysis of the nucleotide sequence of the substitution by splicing site prediction software predicted (with high score, data not shown) the formation of a new donor splice site. To confirm the influence of the transition on the patient's mRNA splicing, we analysed ectopic *F8* transcripts using nested RT-PCR.

After the amplification of exon 8–14 by RT-PCR, Exon 8–11 was amplified using nested primers. The products obtained from 10 independently performed nested PCR using the mRNA prepared by single extraction are shown in Fig. 2. Although the products amplified from each reaction tube were different, overall, three different size RT-PCR products were observed as the products. Nucleotide sequencing of the largest RT-PCR product, detected in seven of 10 reactions, revealed that a 226 bp nucleotide sequence, a part of the intron 10 region, recognized as exon, was inserted between exon 10 and 11 in the mRNA. The nucleotide sequence showed that the middle and small sized

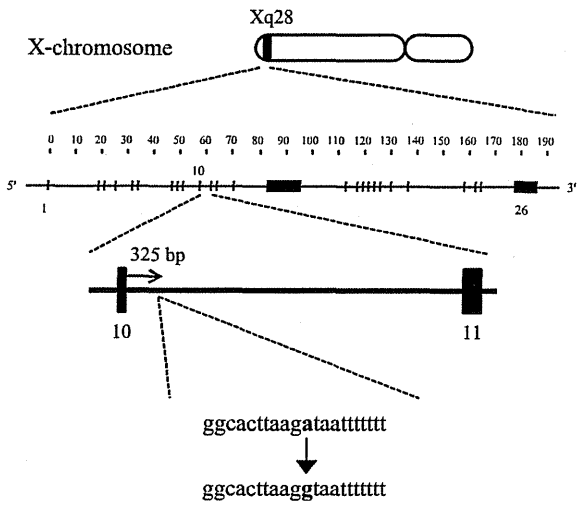

Fig. 1. Adenine to guanine transition in intron 10 of *F8*. The adenine to guanine transition was detected 325 bp downstream from the 3′ end of exon 10 (c.1478 + 325A>G).

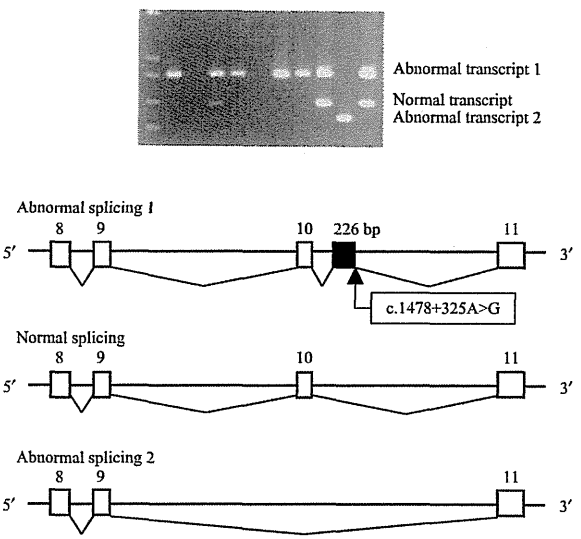

Fig. 2. *F8* mRNA splicing in the patient. The transcript of the patient was analysed by nested RT-PCR. After the amplification of exon 8–14 by RT-PCR, exon 8–11 was then amplified by using nested primers. Two abnormal sized transcripts (abnormal transcript 1 and 2), were detected by 10 independently performed nested PCR from the patient's mRNA prepared by single extraction. Nucleotide sequencing of the abnormal transcript 1 revealed that a 226 bp nucleotide sequence, a part of intron 10, recognized as exon, was inserted between exon 10 and 11 in the mRNA. Nucleotide sequencing revealed that the abnormal transcript 2 was synthesized by exon 10 skipping. The majority of the patient's transcript was abnormal, however, the existence of a small amount of normal transcript was confirmed.

RT-PCR products corresponded to the normal and exon 10-skipping transcripts respectively. These results suggest that the majority of the patient's transcript was abnormal. However, these results also indicated the existence of a small amount of normal transcript.

As the inserted sequence was thought to lead to a frameshift and to generate a premature termination codon in the inserted sequence, it was predicted that degradation of the abnormal mRNA by the mRNA surveillance system (Nonsense-mediated mRNA decay) would occur [12,13]. To estimate the *F8* mRNA expression level, relative quantification analysis using real-time PCR was performed. Two different regions, upstream (exon 1–2) and downstream (exon 20–21) of the transition, were used for amplification. The patient's ectopic *F8* mRNA level was about 1/10 that of the normal Japanese male subjects used as normal controls (Fig. 3). This phenomenon was similar both upstream and downstream of the mutation. These findings suggested that the transition in intron 10 might lead to haemophilia aetiology by decreasing the amount of normal *F8* mRNA.

Characterization of an anti-FVIII antibody

We characterized the anti-FVIII antibody (inhibitor) that developed in the patient. The inhibitor showed high titre (53.2 BUs; Bethesda Units) and a type I inhibition kinetic pattern (data not shown). The predominant IgG subclass was IgG4, with IgG1 present as a minority (data not shown). The epitopes of the inhibitor were both the A2 domain and the light chain (A3-C1-C2 domain) of FVIII (Fig. 4).

Analysis of the genetic risk factors for inhibitor development

The haplotypes of the immune response factor related to risk of inhibitor development were analysed (Table 1). Low risk was suggested in IL10 and TNFα

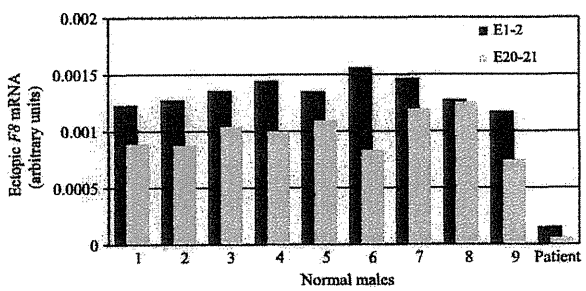

Fig. 3. Ectopic *F8* mRNA quantification. *F8* mRNA levels were relatively quantified by using two different regions, upstream of the transition (exon 1–2) and downstream of the transition (exon 20–21). The result was normalized with endogenous control β-actin and expressed relative to the *F8* level of a male Caucasian liver which was given an arbitrary value of 1. 1–9; Normal Japanese males control.

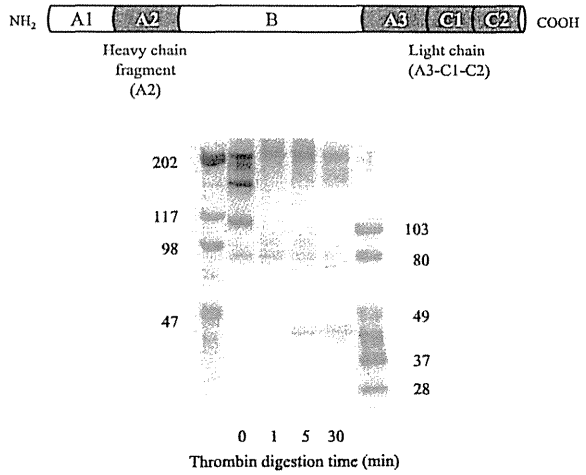

Fig. 4. Inhibitor epitope analysis. The inhibitor recognizes both the A2 domain (44 kDa) and the light chain (A3-C1-C2 domain; 83 kDa) of FVIII.

analysis and high risk was suggested in CTLA-4 analysis. These results suggest that the patient would not be at an especially high risk of inhibitor development.

Discussion

We identified an adenine to guanine transition from deep inside intron 10 of *F8* as a putative cause of mild haemophilia A. The transition, located at 325 bp downstream of exon 10, was found serendipitously because the primer we designed for the amplification of exon 10 was positioned very deep inside intron 10. We usually use the primer which we designed originally for the *F8* analysis. It was difficult to design the primer pair that amplifies exon 10 in our examination. Therefore, as a result of careful selection, the primer positions were decided at deep inside of the intron.

The genetic abnormalities which cause haemophilia A are usually detected in *F8*. However, in about 2% of Haemophilia A patients, no genetic abnormality can be found, even after complete sequencing of *F8* including the promoter and the 3′-UTR regions. Because *F8* is very large, 186 kb long, the range which can usually be analysed is restricted to the coding region including flanking splice sites and is less than one-tenth of the entire *F8* gene. The remainder regions, representing almost all of the intronic sequences, are unanalysed. Therefore, in cases where a gene abnormality has not been detected there is the possibility that some abnormalities are hidden in the intronic regions which remain unanalysed.

The *F8* gene is mainly expressed in sinusoidal endothelial cells and Kupffer cells in the liver [14]. However, trace amount levels of *F8* mRNA, ectopic mRNA, exist in blood cells and can be analysed by RT-PCR amplification [10,15,16]. The analysis of the ectopic

Table 1. Genotypic risk factors for inhibitor development of the patient. The immune response factors related to the risk of inhibitor development were analysed. Low risk was suggested by IL10 and TNFα analysis and high risk was suggested by CTLA-4 analysis.

TNFα
Hap 1 or 3 and -308G/G genotype
(High risk: Hap 2 or 3 and -308G/G genotype)
CTLA-4
–318 C/C
(Low risk: -318 T allele)
IL10
None with allele 134 (138/140)
(High risk: Allele 134 positive)

mRNA obtained from blood is available to observe the state of splicing, and this analysis is widely used to screen for genetic abnormalities. If the mutation exists deep inside the intron, it will give some influence on the transcript. Therefore, examination of the mRNA is very effective to detect unknown genetic mutations or rearrangements. Furthermore, the analysis of ectopic mRNA is also effective to examine the influence that detected gene abnormalities exert on the splice. In fact, the mutation that we found was confirmed to cause the splice abnormality by analysing ectopic mRNA. Although predictive software analysis [17] suggested that this patient's mutation may cause splicing abnormalities, there was no further evidence to prove this.

We analysed ectopic mRNA by using the method that had been reported by El-Maarri *et al.* [10]. This method utilizes the nested PCR technique and is suitable for detection of small amounts of mRNA. At first, the *F8* is divided into four regions, exon 1–8, 8–14, 14–21 and 19–26, and is amplified. Then, each of the first amplification products are further divided into two regions and amplified again. In the present study, because the position of the mutation was identified within intron 10, exon 8–14 was amplified first, then exon 8–11 was amplified. However, consistent amplicons were not obtained from every reaction of the second amplification. We have re-evaluated the preparation of mRNA and the amplification of cDNA to elucidate why the same amplicon was not provided every time. However, we could not resolve the issue. This may reflect the low abundance of such a variant. Therefore, we evaluated the patient's mRNA by the result of 10 independently performed reactions. Although the method mentioned above is an excellent method for analysing ectopic *F8* mRNA, in the case of some splice variants it is suggested that careful evaluation and selection of analyses are necessary.

Originally, the patient was identified as having very mild congenital haemophilia A. The patient had no history of haemorrhage that required treatment until the detection of low FVIII activity level at the age of 60, although he had showed some difficulty of haemostasis, for example in tooth extractions etc. during childhood. The fact that there is agreement between both the FVIII levels at a preoperative examination and the *F8* mRNA levels described in the present study supported the classification of the patient as having mild congenital haemophilia. However, at the present time, the patient has fallen into a very severe state due to development of anti-FVIII antibody. The inhibitor development process of the patient was typical, and took less than 20 exposure days from the first FVIII concentrate injection [18]. Generally, inhibitor development in congenital haemophilia is more frequently observed in the severe patients null mutations [8]. It is comparatively rare that a patient with mild haemophilia A should develop the inhibitor. Inhibitor development in mild haemophilia A is typically observed in patients with molecular abnormalities because endogenous abnormal mutant FVIII, a cross-reacting material (CRM), is recognized as "self" and exogenously infused normal FVIII molecule is recognized as "non-self". The developed antibody is often seen to cross-react with not only "non-self" but also "self".

This patient was diagnosed with congenital mild haemophilia A and has CRM as previously stated. However, analysis of the mRNA might suggest that this patient's CRM would be normal FVIII, produced by the normal mRNA which avoided abnormal splicing. Therefore, this is an interesting case because the inhibitor in this patient raises the possibility that the nature and developing mechanism are different from the inhibitor usually developed in congenital mild haemophilia A. The inhibitor showed a type I inhibition kinetic pattern [19], predominantly IgG4 subclass [20], and multi-clonal epitopes (A2 domain and the light chain of FVIII). These characteristics were most typical of an alloantibody developed in congenital haemophilia. Moreover, we investigated the genetic risk factors in consideration of the possibility that the patient's antibody developed as an autoantibody. The results of the haplotype analysis of the immune response factors suggested that the patient was not at an especially high risk of inhibitor development [21–24]. However, on the other hand, the fact that the patient is aged and had a solid tumour (stomach cancer) may have created a higher risk of an autoantibody development. Therefore, we cannot deny the possibility that the patient's haemophilia is acquired.

The novel factor VIII mutation identified here provides potential insight into the genetic contribution to haemophilia A pathogenesis and inhibitor development. Although the FVIII antibody developed in this patient is interesting, further analysis and knowledge are necessary to judge whether the inhibitor is an alloantibody or an autoantibody.

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Disclosures

K. Fukutake has received speaking fees and honoraria from Baxter Healthcare, Bayer HealthCare and Pfizer Inc. K. Shinozawa is an employee of an endowed chair at the Department of Molecular Genetics of Coagulation Disorders of Tokyo Medical University, which received funding from Baxter Healthcare. The other authors declare that they have no interests that might be perceived as posing a conflict of interest.

References

1 Gitschier J, Wood W, Goralka T *et al.* Characterization of the human factor VIII gene. *Nature* 1984; 312: 326–30.

2 Toole J, Knopf J, Wozney J *et al.* Molecular cloning of a cDNA encoding human antihaemophilic factor. *Nature* 1984; 312: 342–7.

3 Klopp N, Oldenburg J, Uen C, Schneppenheim R, Graw J. 11 hemophilia A patients without mutations in the factor VIII encoding gene. *Thromb Haemost* 2002; 88: 357–60.

4 Vidal F, Farssac E, Altisent C, Puig L, Gallardo D. Rapid hemophilia A molecular diagnosis by a simple DNA sequencing procedure: identification of 14 novel mutations. *Thromb Haemost* 2001; 85: 580–3.

5 Schwartz R, Abildgaard C, Aledort L *et al.* Human recombinant DNA-derived antihemophilic factor (factor VIII) in the treatment of hemophilia A. recombinant Factor VIII Study Group. *N Engl J Med* 1990; 323: 1800–5.

6 Ehrenforth S, Kreuz W, Scharrer I *et al.* Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet* 1992; 339: 594–8.

7 Bray G, Gomperts E, Courter S *et al.* A multicenter study of recombinant factor VIII (recombinate): safety, efficacy, and inhibitor risk in previously untreated patients with hemophilia A. The Recombinate Study Group. *Blood* 1994; 83: 2428–35.

8 Oldenburg J, El-Maarri O, Schwaab R. Inhibitor development in correlation to factor VIII genotypes. *Haemophilia* 2002; 8: 23–9.

9 Astermark J. Overview of inhibitors. *Semin Hematol* 2006; 43: S3–7.

10 El-Maarri O, Herbiniaux U, Graw J *et al.* Analysis of mRNA in hemophilia A patients with undetectable mutations reveals normal splicing in the factor VIII gene. *J Thromb Haemost* 2005; 3: 332–9.

11 Kasper C, Aledort L, Aronson D *et al.* Proceedings: A more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 1975; 34: 612.

12 Amrani N, Sachs M, Jacobson A. Early nonsense: mRNA decay solves a translational problem. *Nat Rev Mol Cell Biol* 2006; 7: 415–25.

13 Chang Y, Imam J, Wilkinson M. The nonsense-mediated decay RNA surveillance pathway. *Annu Rev Biochem* 2007; 76: 51–74.

14 Hollestelle M, Thinnes T, Crain K *et al.* Tissue distribution of factor VIII gene expression in vivo–a closer look. *Thromb Haemost* 2001; 86: 855–61.

15 Bidichandani S, Lanyon W, Shiach C, Lowe G, Connor J. Detection of mutations in ectopic factor VIII transcripts from nine haemophilia A patients and the correlation with phenotype. *Hum Genet* 1995; 95: 531–8.

16 Maugard C, Tuffery S, Aguilar-Martinez P *et al.* Protein truncation test: detection of severe haemophilia a mutation and analysis of factor VIII transcripts. *Hum Mutat* 1998; 11: 18–22.

17 Reese M, Eeckman F, Kulp D, Haussler D. Improved splice site detection in Genie. *J Comput Biol* 1997; 4: 311–23.

18 Dimichele D. Inhibitors: resolving diagnostic and therapeutic dilemmas. *Haemophilia* 2002; 8: 280–7.

19 Biggs R, Austen D, Denson K, Borrett R, Rizza C. The mode of action of antibodies which destroy factor VIII. II. Antibodies which give complex concentration graphs. *Br J Haematol* 1972; 23: 137–55.

20 Gill J. The role of genetics in inhibitor formation. *Thromb Haemost* 1999; 82: 500–4.

21 Astermark J, Oldenburg J, Pavlova A, Berntorp E, Lefvert A, MIBS Study Group. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood* 2006; 107: 3167–72.

22 Astermark J, Oldenburg J, Carlson J *et al.* Polymorphisms in the TNFA gene and the risk of inhibitor development in patients with hemophilia A. *Blood* 2006; 108: 3739–45.

23 Astermark J, Wang X, Oldenburg J, Berntorp E, Lefvert A, MIBS Study Group. Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. *J Thromb Haemost* 2007; 5: 263–5.

24 Pavlova A, Delev D, Lacroix-Desmazes S *et al.* Impact of polymorphisms of the major histocompatibility complex class II, interleukin-10, tumor necrosis factor-alpha and cytotoxic T-lymphocyte antigen-4 genes on inhibitor development in severe hemophilia A. *J Thromb Haemost* 2009; 7: 2006–15.

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Author Contribution

SFP and AS performed the laboratory experiments; SMR and MCO designed the research study; BVC, SMR, SFP, MCO and DZ collected the clinical data; CC, MCO and SMR analysed the data; MCO, SMR, GCO and SFP wrote the paper.

Disclosures

The authors state that they had no interests which might be perceived as posing a conflict or bias.

References

1 Graw J, Brackmann HH, Oldenburg J, Schneppenheim R, Spannagl M, Schwaab R. Haemophilia A: from mutation analysis to new therapies. *Nat Rev Genet* 2005; 6: 488–501.
2 Gouw SC, van den Berg HM. The multifactorial etiology of inhibitor development in haemophilia: genetics and environmental. *Semin Tromb Hemost* 2009; 35: 723–34.
3 Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factors VIII and IX. *Haemophilia* 2006; 12(Suppl. 6): 15–22.
4 Margaglione M, Castaman G, Morfini M *et al.* The Italian AICE-Genetics hemophilia A database: results and correlation with clinical phenotype. *Haematologica* 2008; 93: 722–8.
5 Antonarakis SE, Rossiter JP, Young M *et al.* Factor VIII gene inversion in sevee hemophilia A: results of an international consortium study. *Blood* 1995; 86: 2206–12.
6 Leiria L, Roisenberg I, Salzano F, Bandinelli E. Intron 1 and 22 invrsions and factor VIII inhibitors patients with severe haemophilia A in southern Brazil. *Haemophilia* 2009; 15: 309–13.
7 Gouw Sc, Van Der Bom JG, Van Den Berg HM *et al.* Influence of the type F8 gene mutation on inhibitor development in a single centre cohort of severe haemopilia A patients. *Haemophilia* 2011; 17: 275–81.
8 Viel KR, ameri A, Abshire TC *et al.* Inhibitors of Factor VIII in black patients with hemophilia. *N Engl J Med* 2009; 360: 1618–27.
9 Santos A, Annichino-Bizzacchi JM, Ozelo MC. Inhibitors of factor VIII in hemophilia. *N Engl J Med* 2009; 361: 309–10.

Arthroscopic synovectomy of the elbow covered with rFVIIa in a haemophilia B juvenile with inhibitor

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We report a successful case of arthroscopic synovectomy for both elbows in a haemophilia B juvenile with inhibitor covered with activated recombinant factor VII (rFVIIa).

An inhibitor haemophilia B juvenile (16 years, 50 kg of body weight) was suffering from intra-articular bleeding once or twice a month for years with unbearable pain in both elbows, which was followed by upper limbs dysfunction. In 2008, his right elbow showed mild contracture resulting in some limitation of daily activity. His left elbow showed better range of motion, however, it was strictly limited for several days after haemarthrosis resulting in more severe limitation of activity than the right (see Table 1). The left elbow was attacked more frequently than the right in those days. Radiographs showed signs of moderate degenerative arthritis including epiphysis enlargement of the radial head (Arnold-Hilgartener stage IV [1]) on the right elbow and slight erosion in the articular surface on the left elbow (Arnold-Hilgartener stage III). MRI showed hypertrophy of synovium with pooling of joint effusion (Fig. 1).

He had been diagnosed as haemophilia B because of intracranial haemorrhage in new born and high responded inhibitor of factor IX developed when he was 1-year old. After that intra-articular bleeding occurred in his several joints including both elbows. Replacement therapy by rFVIIa improved his joints' disorders, although some target joints still existed. Before surgery, activated partial thromboplastin time (APTT) was prolonged to 123.0 s and factor IX inhibitor was 68 BU mL^{-1}. Preoperative rFVIIa trial revealed effective shortening of the prothrombin time [PT-INR was 1.15 before and was kept from 0.66 to 0.68 for 2 h after intravenous injection of 6.0 mg (120.0 $\mu g\ kg^{-1}$) of rFVIIa].

We had planned arthroscopic synovectomy on his left elbow at first. On the day of the operation, pre- and intraoperative bolus infusion of 6.0 mg of rFVIIa was administered every 2 h. Congestive hypertrophic synovium was successfully removed by arthroscopy. Bolus infusion dose of 6.0 mg was administered at 2–12 h interval from the operative day 0 to day 8. Careful assistive motion exercise was started on postoperative day 3. He was also instructed to move his elbow with protection by the hinge brace for 3 months. His elbow showed gradual functional improvement without any clear sign of periarticular bleeding. At postoperative 24 months, the left elbow showed painless good function of 135° of flexion with 20° of

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Table 1. Clinical and radiographic status of the patient's both elbows before and after the operation. Preoperative and postoperative status of the patient's both elbows.

		Intra-articular bleeding Number of episodes	Pain	Range of motion (degrees) Flexion	Extension	Pronation	Supination	Radiography Arnold-Hilgartener stage
L) Elbow	Preop.	10–15/Years	Moderate	130/110*	−20/−25*	80	90	III
	Postop. (36 months)	None	None	135	−20	80	80	II
R) Elbow	Preop.	Several times/Year	Mild	120	−30	45	60	IV
	Postop. (12 months)	None	None	135	−30	60	70	IV

*Preoperative range of motion of left elbow was reduced in several days after intra-articular bleeding.

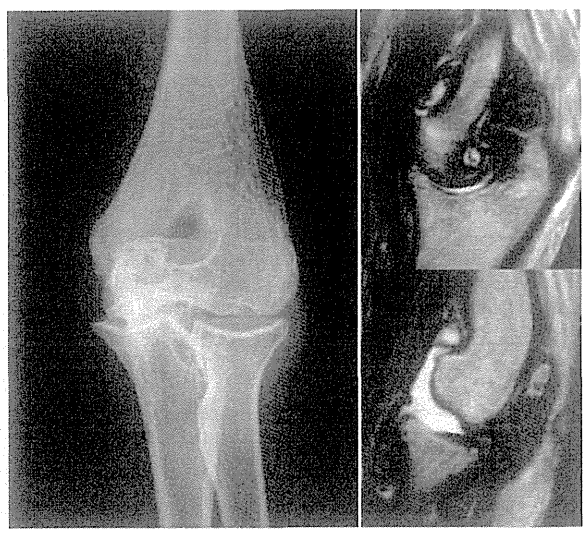

Fig. 1. Preoperative AP radiogram and MRI T2-weighted sagital images of the left elbow show erosive change on the articular surface, hypertrophy of synovium and correction of joint effusion.

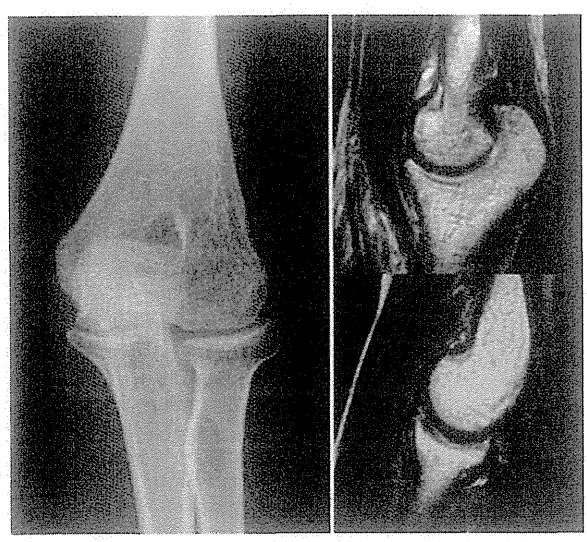

Fig. 2. AP radiogram and MRI T2-weighted sagital images of the left elbow obtained 2 years after the arthroscopic synovectomy show good congruity of the joint with remodelling of subchondral bone. No synovitis exists.

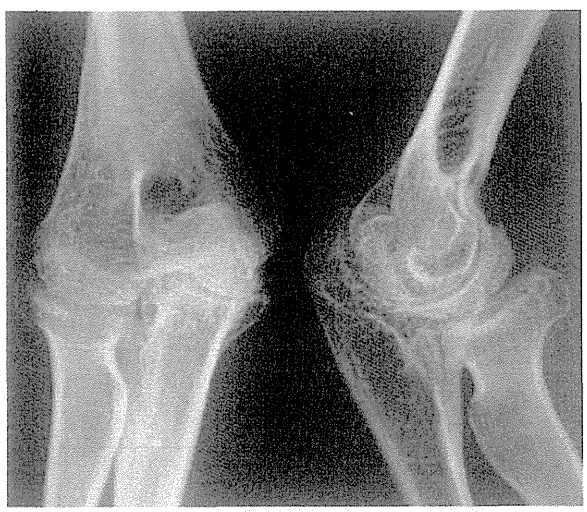

Fig. 3. Radiograms of the degenerative arthritic right elbow before the operation.

extension loss. The radiographs showed improvement of the subchondral erosion (Arnold-Hilgartener stage II) and MRI showed reduction in joint effusion (Fig. 2), however, the right elbow dysfunction slightly progressed with a few episodes of haemarthrosis (Fig. 3).

Arthroscopic synovectomy of the right elbow with shaving of the deformed radial head was performed under the same protocol. Postoperative bleeding was controlled successfully and the motion exercise advanced without complication. He regained good right elbow function quickly after the procedure (see Table 1). Now it is 3 years for his left elbow and 1 year for his right elbow after each of the operation. He can do all activities of daily living without any episode of intra-articular bleeding. Good remodelling of the articular congruity without progression of degeneration was observed in postoperative radiograms (Fig. 4).

Discussion

Elbow is a key joint of the upper limb function and limitation of elbow motion is critical if flexion is limited under 130°, extension under −30° (flexion contracture) and prono-supination under 50° [2]. It is often involved as a target joint in haemophilia and repeating intra-articular bleeding results in an arthritic elbow, which limits the patients' daily activity [3]. Synovectomy for haemophilic arthropathy is effective to reduce pain and frequency of bleeding, but conventional open surgery was so invasive that postoperative joint contracture might cause deteriorative effect for function. Especially in an inhibitor patient, surgical intervention was still challenging.

Arthroscopy was developed in 1940s and has been applied as a tool of less-invasive surgery for haemophilic arthropathy in comparatively large joints such as the knee [4]. This procedure may

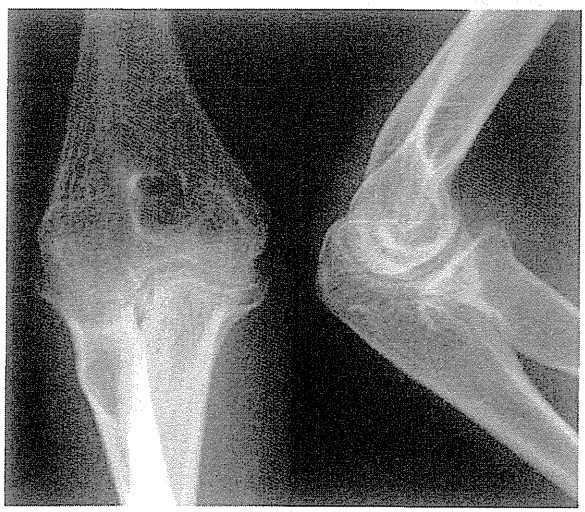

Fig. 4. Radiograms obtained postoperative 12 months. Better congruity was obtained after arthroscopic shaving of the radial head.

improve the stage of haemophilic arthropathy especially in young patients who have the potential of osteochondral remodelling [5], however, it is not popular for the elbow because of technical difficulty; the joint cavity is small and close to the major neurovascular structures. But technical development enabled arthroscopic elbow surgery possible and popular [6]. In medication, development of bypassing agents, such as activated prothrombin complex concentrate (aPCC), and activated recombinant factor VII (rFVIIa) made it possible to apply surgery for an inhibitor patient of haemophilia [7,8].

Our patient was a juvenile with high responded inhibitor and just lost his elbow function because of early or moderate stage of haemophilic arthropathy. Arthroscopic elbow synovectomy w/o radial head shaving was successfully performed by covering with rFVIIa, which resulted in not only good control of articular bleeding but also functional improvement. Radiographic improvements in his left elbow suggest the effectiveness of this approach, especially for young less-progressive haemophilic arthropathy. Even in more degenerative right elbow, functional improvement was achieved after shaving of the radial head. In the past, arthroscopy was not popular for the elbow because the joint cavity is small and close to major neurovascular structures. Technical developments have recently enabled more successful arthroscopies of the elbow. Haemophilic arthropathy may be improved especially in young patients with the potential of osteochondral remodelling. Use of rVIIa enabled us to perform two such procedures in a patient with an inhibitor to factor IX.

Disclosures

KS and KI received reimbursement for attending a symposium of haemophilia research meeting and also received a fee for speaking at the meeting from the Novo Nordisk Pharma Ltd.

References

1 Arnold WD, Hilgartner MW. Hemophilic arthropathy. *J Bone Joint Surg Am* 1977; 59: 287–305.

2 Morrey BF, An K-N. Functional evaluation of the elbow. In: Morrey BF ed. The Elbow and its Disorders, 3rd edn. Philadelphia, United States: WB Saunders Ltd, 2000: 74–83.

3 Gamble JG, Vallier H, Rossi M, Glader B. Loss of elbow and wrist motion in hemophilia. *Clin Orthop* 1996; 328: 94–101.

4 Wiedel JD. Arthroscopic synovectomy for chronic hemophilic synovitis of the knee. *Arthroscopy* 1985; 1: 205–9.

5 Tanaka Y, Shinohara Y, Narikawa K *et al.* Arthroscopic synovectomies combined with reduced weight-bearing using patella tendon-bearing braces were very effective for progressed haemophilic ankle arthropathy in three paediatric patients. *Haemophilia* 2009; 15: 833–6.

6 Poheling GG, Whipple TL, Sisco L, Goldman B. Elbow arthroscopy: a new technique. *Arthroscopy* 1989; 5: 222–4.

7 Hay CR, Brown S, Collins PW, Keeling DM, Liesner R. The diagnosis and management of factor VIII and IX inhibitors: a guideline from the United Kingdom haemophilia canter doctors organization. *Br J Haematol* 2006; 133: 591–605.

8 Takedani H, Kawahara H, Kajiwara M. Major orthopaedic surgeries for haemophilia with inhibitors using rFVIIa. *Haemophilia* 2010; 16: 290–5.

Immune system polymorphisms and factor VIII inhibitor formation in Brazilian haemophilia A severe patients

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The main problem facing the replacement therapy of severe haemophilia A patients is the development of factor VIII (FVIII) inhibitors, which occur in 10–30% of them. Type of the therapeutic treatment, age of its onset and the frequency of its administration clearly influence inhibitors formation. But the type of FVIII gene mutation they have, and variables of their immune system also play a role. In this article, we approached the investigation of these variables by considering 15 polymorphisms related to five systems: (i) HLA-G, the non-classical class 1 human leucocyte antigen; (ii) PTN22, a tyrosine phosphatase that negatively regulates stimulatory signalling cascades; (iii) IL4 and IL10, interleukins 4 and 10, soluble

基礎・診療の基礎

治療歴のある血友病B患者における血漿由来血液凝固第Ⅸ因子製剤（ノバクト®M静注用）の生体内回収率

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瀧　　正志[1]

要　　旨

治療歴のある血友病B患者において血漿由来モノクローナル抗体精製血液凝固第Ⅸ因子製剤（ノバクト®M静注用）の薬物動態について検討すると共に，年齢，体重，ヘマトクリット（Hct）値およびbody mass index（BMI）が生体内回収率に影響を与えるか否かについて検討した。その結果，ノバクトM静注用を投与した30分後の生体内回収率は51.8±15.0%であり，第Ⅸ因子活性上昇値（IVR）は1.04±0.30（U/dL per IU/kg）であった。また，年齢による生体内回収率の違いを検討した結果，16歳未満の回収率は44.2±8.9%であったのに対し，16歳以上の回収率は61.3±16.8%であり，両者は大きく異なっていた。更に，体重とBMIは，生体内回収率と有意な正の相関が確認されたが，Hct値は回収率と有意な相関がなかった。

今回の検討におけるノバクトM静注用の生体内回収率は，海外で報告されている血漿由来第Ⅸ因子製剤の回収率とよく一致していた。今回の検討では，生体内回収率に個人差があること，更に同じ患者でも回収率に変動があることが確認されたので，個々の患者で回収率を適宜測定し，その結果を基に適切な投与量を設定することが重要である。

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Recovery of the Plasma-Derived Factor Ⅸ Concentrate (Novact® M) in Previously Treated Patients with Hemophilia B

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はじめに

血友病Bは，血液凝固第Ⅸ因子が欠乏することによる稀な遺伝性の出血性疾患である[1]。この血友病Bの止血管理には，主に血液凝固第Ⅸ因子製剤が使用されており，国内では血漿由来モノクローナル抗体精製第Ⅸ因子製剤と遺伝子組換え第Ⅸ因子製剤が主として使用されている[2]。

血液凝固第Ⅸ因子製剤の薬物動態は，製剤によって異なることが報告されており，特に血漿由来第Ⅸ因子製剤と遺伝子組換え第Ⅸ因子製剤で大きく異なっていることが報告されている[3)4)]。血漿由来第Ⅸ因子製剤を患者に投与した時の生体内回収率は，約50%程度であるが，遺伝子組換え第Ⅸ因子製剤の場合には約35%と報告[3]されている。

また，血友病Bの患者毎に生体内回収率が異なることや，成人よりも小児の生体内回収率が低いことが報告[5]されている。同じ患者でも，加齢により生体内回収率が変動することや，加齢以外でも回収率の変動があることが報告[6]されている。

国内では，血漿由来の第Ⅸ因子製剤が3製剤，遺伝子組換え第Ⅸ因子製剤が1製剤の計4製剤が血友病Bの止血管理に使用できる。ノバクト®Mは，1992年の発売より血友病Bの止血管理に使用されているモノクローナル抗体で精製された血漿由来第Ⅸ因子製剤である。このノバクトMは，2010年6月に国際単位で表示された製剤（ノバクト®M静注用400単位・800単位・1600単位，以下ノバクトM静注用と略す）に変更されたが，その国際単位表示の製剤による生体内回収率のデータは乏しいのが現状である。そこで，当施設に通院している血友病B患者でノバクトM静注用の薬物動態について検討すると共に，年齢，体重，ヘマトクリット（Hct）値およびbody mass index（BMI）が生体内回収率に影響を与えるか否かについて検討した。

Ⅰ 試験対象

対象は，当施設に通院している血友病B患者9例で，年齢は平均15.7歳（1～34歳）で16歳未満が5例，16歳以上が4例であった。

Ⅱ 試験方法

1．生体内回収率の測定

生体内回収率は，前回の第Ⅸ因子製剤の投与から3日以上の間隔を空け，かつ出血がないことを確認して測定した。ノバクトM静注用は，患者の体重kg当たり50単位（IU）を目安に投与し（平均投与量48.7IU/kg），投与前と投与30分後の採血で第Ⅸ因子活性を測定した。生体内回収率は，投与30分後における第Ⅸ因子活性の実測上昇値を体重kg当たりの投与量の2倍で除して求めた。計算式を以下に示す。

表1　生体内回収率と第Ⅸ因子活性上昇値（IVR）

患者番号	投与量 (IU/kg)	生体内回収率 (%)	第Ⅸ因子活性上昇値 (U/dL per IU/kg)
1	53.3	39.3	0.79
2	84.5	33.0	0.66
3	46.5	45.2	0.90
4	29.8	47.0	0.94
5	48.9	82.9	1.66
6	43.0	65.4	1.31
7	53.1	44.8	0.90
8	54.9	51.9	1.04
9	24.6	56.6	1.13
平均	48.7	51.8	1.04
標準偏差	17.1	15.0	0.30
95%信頼区間	42.0～61.6	42.0～61.6	0.84～1.24

生体内回収率（%）＝

第Ⅸ因子活性の実測上昇値÷投与量(IU/kg)÷2×100

2．第Ⅸ因子活性上昇値（IVR, *in vivo* recovery）の計算

第Ⅸ因子活性上昇値（IVR）は，下記の式のように投与30分後の第Ⅸ因子活性の実測上昇値（%＝U/dL）を体重kg当たりの第Ⅸ因子製剤投与量（IU/kg）で除して求めた。

第Ⅸ因子活性上昇値（IVR）＝

第Ⅸ因子活性の実測上昇値（U/dL）÷第Ⅸ因子製剤投与量（IU/kg）

Ⅲ　結果および考察

1．生体内回収率と第Ⅸ因子活性上昇値（IVR）

ノバクトM静注用を投与した30分後の生体内回収率は51.8±15.0%（95%信頼区間；42.0～61.6）であり，第Ⅸ因子活性上昇値（IVR）は1.04±0.30（U/dL per IU/kg）（95%信頼区間；0.84～1.24）であった（表1）。この結果は，海外で報告[3)4)]されている血漿由来第Ⅸ因子製剤の成績とよく一致した成績である。特にPoonら[5)]は，血漿由来第Ⅸ因子製剤の第Ⅸ因子活性上昇値（IVR）が1.06±0.26（U/dL per IU/kg）と報告しており，今回の値と一致している。

2．年齢による生体内回収率の違い

年齢による生体内回収率の違いを検討するために，16歳未満と16歳以上の2群に分け，比較した（表2および図1）。16歳未満の生体内回収率は，44.2±8.9%であり，16歳以上の回収率は61.3±16.8%であった。16歳以上の生体内回収率を100%とした時に16歳未満の回収率は72%であり，両者は大きく異なっていた。更に，両者の生体内回収率の変動係数（CV）は，20.0%（16歳未満）と26.1%（16歳以上）と大きく，個々の患者毎に回収率が異なることが確認された。これらの結果は，これまでに報告されている小児の生体内回収率が成人に比べて低いこと[5)]，回収率の個人差が大きいこと[7)]とよく一致している。第Ⅸ因子は，生体内に投与された後，速やかに血管内皮細胞に結合するため[8)9)]，生体内回収率の個人差は，血管内皮細胞に対する親和性の差に関連する可能性も示唆されている[10)]。