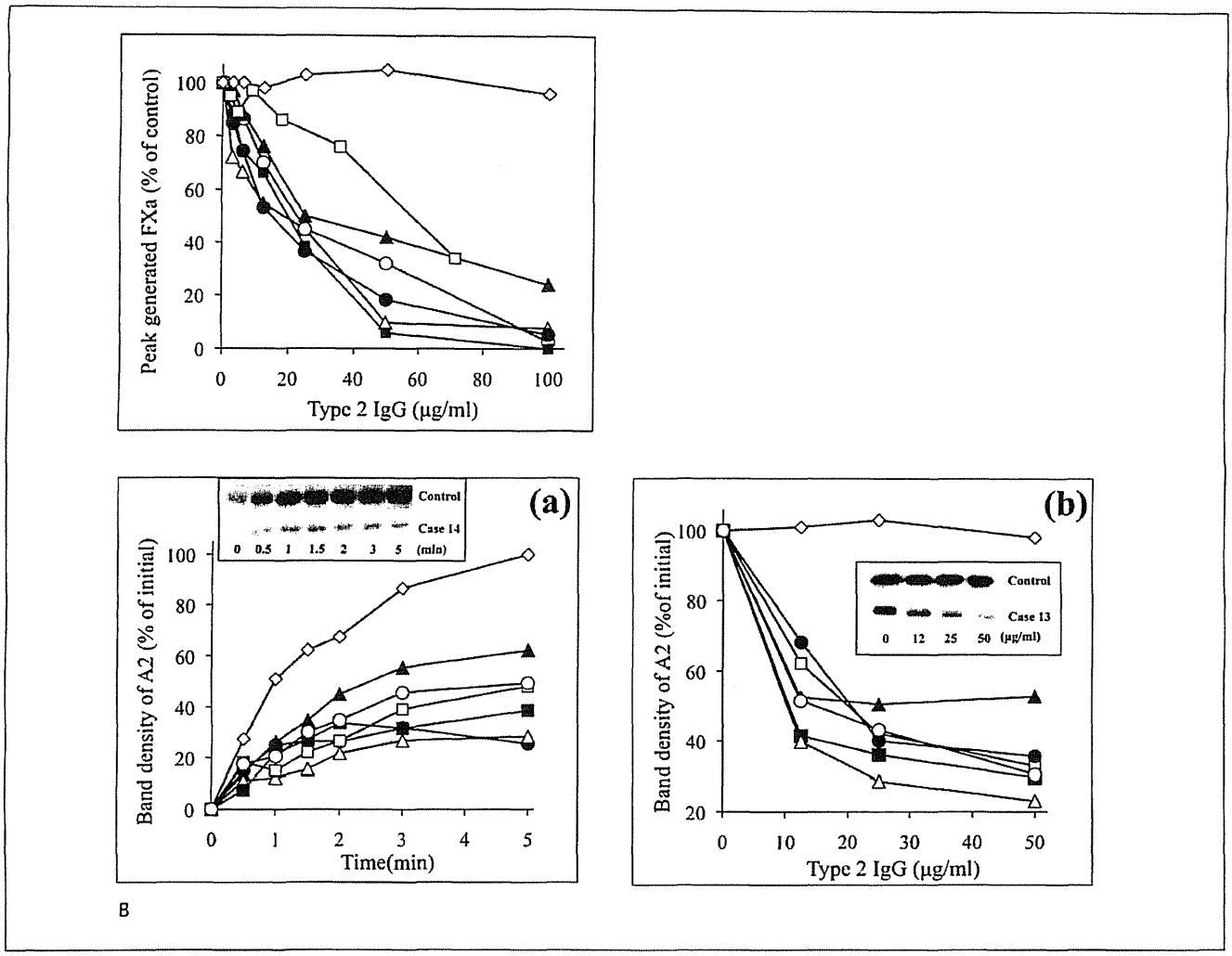

Figure 3: Continued

were in keeping with those observed using anti-C2 AHA plasmas in thrombin and FXa generation assays (see ▶Fig. 2A and B). No significant differences were observed between ESH4 and ESH8 in these assays, however.

Effect of anti-FIX mAb on TGT in FVIII-deficient plasma

Our findings suggested that the additional decrease of coagulation function in AHA relative to S-group could be attributed to the markedly decreased activity of the intrinsic tenase complex. Since intrinsic tenase activity in S-group HA depends on FIXa-catalysed FX activation, we hypothesised that inhibition of FIXa-induced FX activation could have mediated the significantly greater decrease in tenase activity observed in AHA. We examined, therefore, the effects of anti-FIX mAb on thrombin generation in FVIII-deficient plasmas (▶Fig. 5). Control experiments demonstrated that the addition of anti-FVIII alloAb (10 BU/ml) to FVIII-deficient plasmas resulted in similar TGT parameters compared to its absence, confirming complete FVIII deficiency in the plasma samples. Furthermore, the addition of anti-C2 autoAbs to FVIII-deficient plasma little affected thrombin generation (data not shown), confirming that the effects of anti-C2 autoAbs in AHA patients depended on the presence of FVIII.

In addition, anti-FIX mAb3A6 (10 BU/ml) was incubated with FVIII-deficient plasmas, and TGT assays performed as above. Peak thrombin levels in the presence of anti-FIX mAb were significantly more decreased (~1.3-fold) than its absence (8.0 ± 0.5/10.4 ± 0.6 nM, $p<0.05$, ▶Fig. A). Similarly, ETP was more depressed (~1.5-fold) in the presence of anti-FIX mAb than its absence (100 ± 10/144 ± 15 nM, $p<0.05$, ▶Fig. 5C), and the time to peak was prolonged by ~1.3-fold (32.7 ± 1.9/25.0 ± 1.8 min, $p<0.05$, ▶Fig. 5B). These findings were similar to those obtained with native AHA plasmas, and the results were consistent with the concept that the exacerbated haemorrhagic symptoms in AHA with anti-C2 autoAbs, compared to S-group, could be related, in part, to indirect

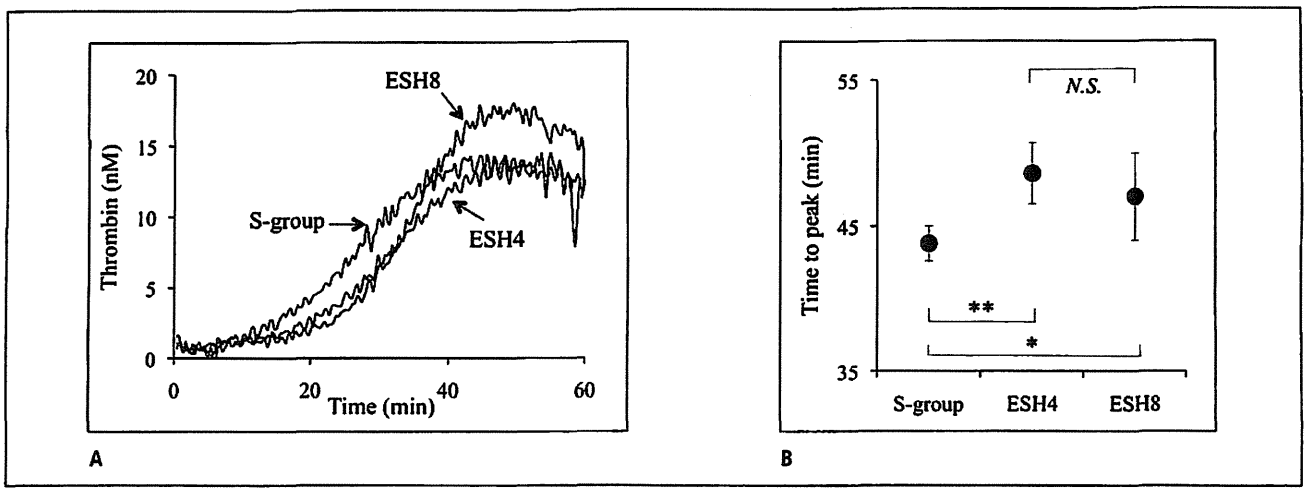

Figure 4: Coagulation function in *in vitro* AHA-models reconstituted with FVIII and anti-C2 mAb. A) FVIII (0.1 nM) was mixed with anti-C2 mAb ESH4 (80 μg/ml) or mAbESH8 (20 μg/ml) for 1 h prior to incubation with FVIII-deficient plasma. Samples were mixed with TF (0.5 pM), PL (60 μM), and ellagic acid (0.3 μM), followed by the addition of $CaCl_2$. Thrombin generation was measured as described in *Methods*. Representative TGT curves were illustrated. B) The time to peak obtained from the TGT is shown in (A). Data are shown as mean ± SD for data from at least five separate experiments. *p<0.05, **p<0.01, NS; no significance.

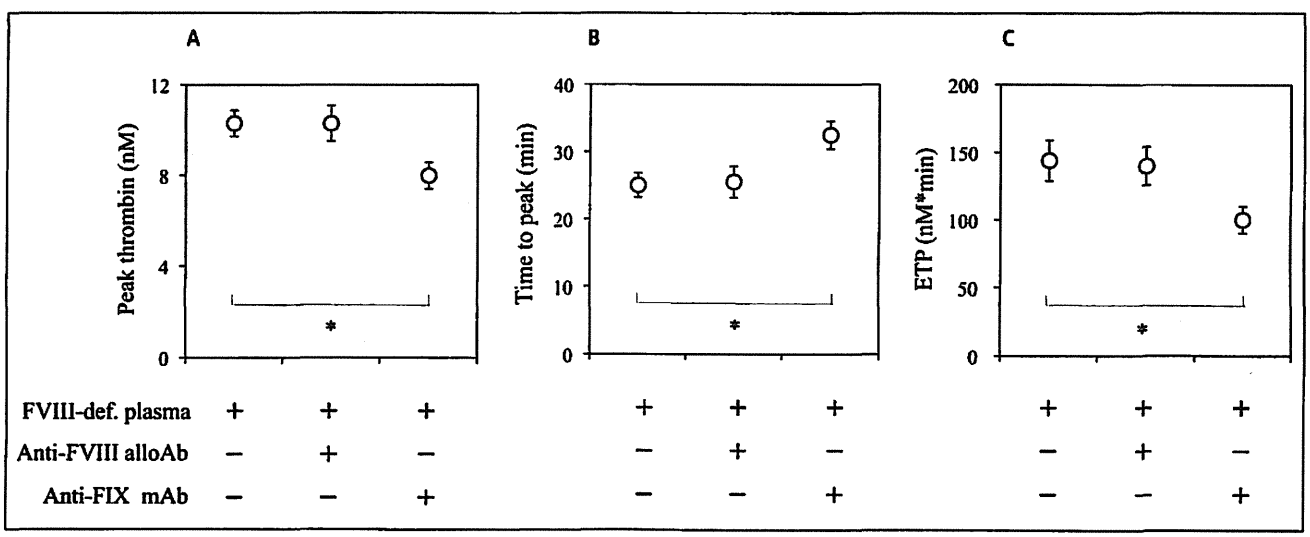

Figure 5: Effect of anti-FIX mAb on the thrombin generation in FVIII-deficient plasmas. FVIII-deficient plasma was preincubated with or without anti-C2 alloAb (10 BU/ml) for 1 h, and was reacted with or without anti-FIX Ab (10 BU/ml) for 1 h. These samples were reacted with TF (0.5 pM), PL (60 μM), and ellagic acid (1.8 μM), followed by the addition of $CaCl_2$. Thrombin generation was measured as described in *Methods*. A-C) Parameters of peak thrombin, time to peak, and ETP obtained from TGT. In all instances, results are shown as mean ± SD from at least five separate experiments. *p<0.05.

inhibition of FIXa-catalysed FX activation due to steric hindrance in the presence of the FVIII-anti-C2 autoAbs complex.

Discussion

The reason(s) why haemorrhagic symptoms in AHA are more severe than those in severe HA, although FVIII:C levels are similar, have not been clarified. The present findings suggest for the first time, that the mechanisms involved in these circumstances could possibly be attributed to the inhibition of FIXa-mediated FX activation by disturbances (steric hindrance) on the tenase complex in the presence of FVIII-anti-C2 autoAb complexes.

AHA antibodies with anti-A2 epitopes were not available for study, and all anti-FVIII autoAbs used in this study recognised the C2 domain. All anti-C2 Type 1 antibodies blocked FVIII binding to VWF and PL, but did not affect FVIII activation by thrombin. In

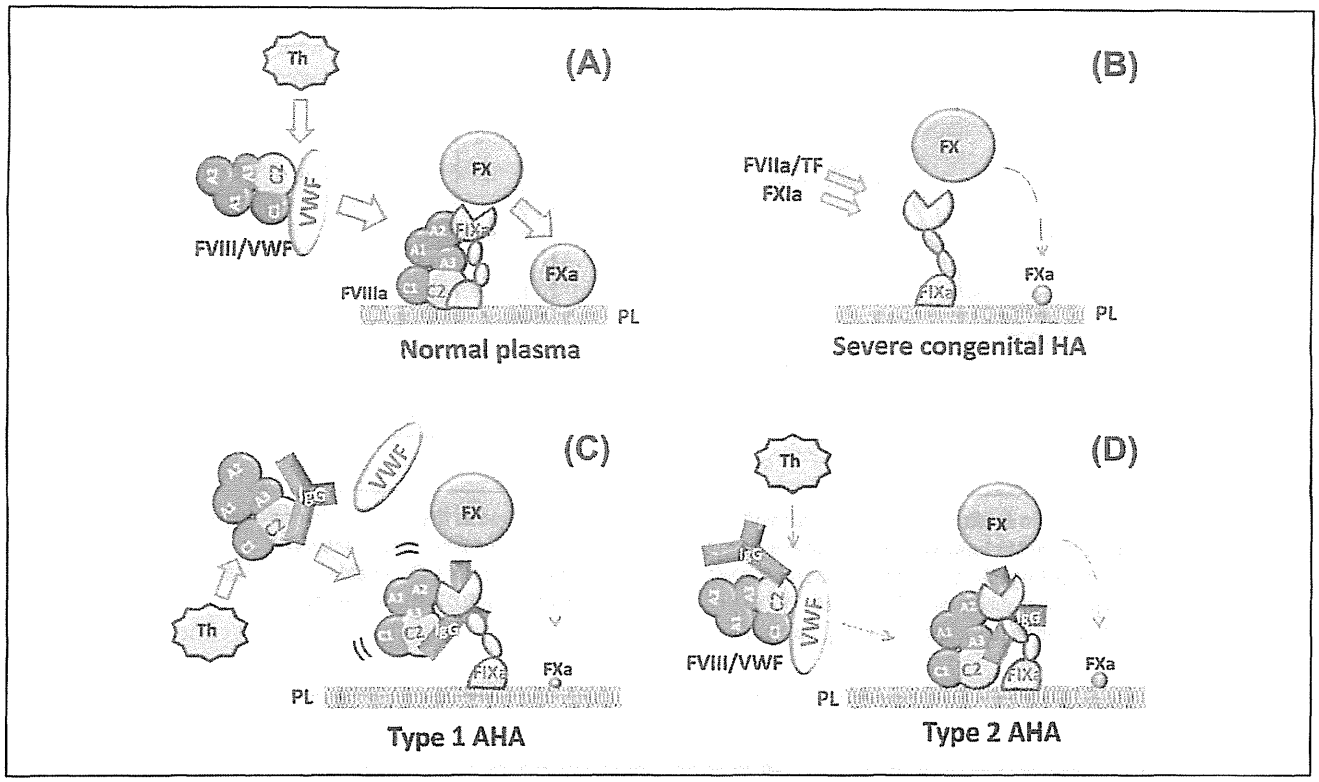

Figure 6: A putative coagulation mechanism for the intrinsic tenase complex in patients from the S-group, anti-C2 type 1, and type 2 AHA. In normal plasmas, free FVIIIa is generated from FVIII/VWF by thrombin, followed by FVIIIa/FIXa-dependent FX activation on PL micelles (A). In severe HA, FIXa alone generates FXa from FX very slowly (panel B). In both type 1 and type 2 AHA, anti-C2 IgG-FVIIIa complexes interfere with FIXa-catalysed FX activation on PL by steric hindrance. In type 1 cases, this complex fails to bind to PL, and the tenase assembly is unstable (C). In type 2 cases, although anti-C2 IgG significantly blocks thrombin-induced FVIII activation, small amounts of the FVIIIa-IgG complex bind to PL, and consequently trace amounts of tenase assembly is formed (D).

contrast, all anti-C2 type 2 antibodies inhibited FVIII activation by thrombin, but did not affect FVIII binding to VWF and PL. These anti-C2 properties were similar to those reported by Meeks et al. (9, 10), and were representative of the classical and non-classical anti-C2 antibodies respectively. In addition, PL concentrations did not affect the difference between both groups in thrombin and FXa generation and binding assays (data not shown). SDS-PAGE and Western blotting analysis revealed that the inhibition of thrombin-catalysed FVIII activation by anti-C2 Type 2 was attributed to delayed cleavage at Arg^{372} and Arg^{1689}. It was of additional interest, that mAbESH8 with type 2 epitopes did not affect FVIII cleavage by thrombin at Arg^{372} (and Arg^{1689}) (data not shown, [35]), and the findings might have reflected a novel inhibitory mechanism for anti-C2 autoAb inhibitors. The C2 domain is structurally juxtaposed to the A1 domain (36), and inhibition of cleavage by anti-C2 type 2 may have been due to a polyclonal, steric effect of the anti-C2 autoAbs, although inhibition caused by the coincident presence of an anti-A2 autoAb (37) could not be excluded. We have recently demonstrated an interaction between the C2 domain (residues 2228–2240) and the FIXa Gla domain in the tenase complex (38). In the current studies, however, neither type 1 nor type 2 anti-C2 autoAbs inhibited C2 binding to FIXa (data not shown), suggesting that these antibodies had little direct affect on FVIIIa-FIXa interactions in the tenase complex.

Thrombin generation in AHA was significantly less than that in severe HA. Furthermore, intrinsic FXa generation in AHA, reflecting processes upstream of thrombin generation, was decreased relative to that in severe HA. The anti-C2 antibodies little inhibited prothrombinase activity (data not shown), it appeared, therefore, that critical differences between AHA and severe HA in the intrinsic tenase complex contributed to the clinical findings, and that these differences centered on the effects of anti-C2 autoAbs on FVIIIa, FIXa, FX, and PL interactions. In normal tenase reactions (▶ Fig. 6A), FVIII in complex with VWF, is converted to FVIIIa by thrombin and dissociated from VWF (39). FIXa (activated by FVIIa/TF and/or FXIa) together with FVIIIa, activates FX on PL-membrane surfaces, resulting in FXa generation. In severe HA in the absence of FVIIIa cofactor (▶ Fig. 6B), FX is slowly converted to FXa by FIXa. Our studies demonstrated that FIXa-mediated FX activation in the presence of anti-FIX mAb, or in AHA-models constructed with anti-C2 mAbs, was less effective than that in severe HA. Based on these data, therefore, we propose a putative mechanism for the markedly decreased coagulation function in AHA with anti-C2 autoAbs. We suggest that the anti-C2 autoAbs,

What is known about this topic?

- Acquired haemophilia A (AHA) is caused by the development of factor (F)VIII autoantibodies (autoAbs).
- AHA results in more serious haemorrhagic symptoms than in congenital severe HA, but the reason(s) remain unknown, however.

What does this paper add?

- Coagulation functions, assessed using the global coagulation assays, were significantly more depressed in AHA with anti-C2 autoAbs relative to congenital HA.
- As one of putative mechanism(s), we proposed that the FVIII/anti-C2 autoAb complexes appeared to interfere with FIXa-dependent FX activation indirectly due to steric hindrance.
- In addition, the anti-C2 autoAbs with type 1 behavior prevented FVIII(a)-phospholipid binding mechanisms, essential for the tenase complex, and those with type 2 behaviour decreased the FXa generation by inhibiting thrombin-catalysed FVIII activation, suggesting that these distinct mechanisms could be associated with the exacerbated haemorrhagic symptoms in AHA.

complexed with FVIIIa, indirectly interfere with the association between FIXa and FX on PL-membrane surfaces by steric hindrance. Consequently, FIXa-mediated activation of FX in these patients is depressed to a greater extent than in severe HA (▶Fig. 6C and D).

The assays of thrombin and FXa generation showed that critical coagulation functions in AHA type 1 were lower than those in type 2, and experiments using AHA-models containing anti-C2 mAbs with type 1 and 2 behaviour (ESH4 and ESH8) demonstrated a similar tendency. Both native anti-C2 type 1 autoAbs and ESH4 inhibit FVIII binding to VWF and PL, and this inhibition of VWF-binding would lead to significantly decreased levels of FVIII:C (2). Furthermore, although FVIII-IgG complexes can be completely activated by thrombin, the tenase complex failed to bind to PL-membranes in these circumstances, and the conformation of this complex would likely be extremely unstable (▶Fig. 6C). In contrast, our experiments with native anti-C2 type 2 autoAbs and ESH8 demonstrated that FVIII binding to VWF or PL was little inhibited. It appeared, therefore, that these autoAbs significantly inhibited FVIII activation by thrombin, but that the relatively small amounts of FVIIIa-IgG complex formed bound to PL, facilitating trace amounts of tenase assembly (▶Fig. 6D). Nevertheless, as with type 1 antibodies, indirect disturbances (steric hindrance) mediated by FVIIIa-IgG complexes would have inhibited FIXa-induced FX activation. We speculate, therefore, that differences in the inhibitory mechanisms between type 1 and type 2 antibodies might have contributed to the observations that coagulation parameters were depressed in the order type 1, type 2, and S-group patients. Further studies are required to clarify these mechanisms.

In view of our findings that the excessive decrease in coagulation function in AHA could be due to indirect inhibition of FIXa-dependent FX activation, it might be expected that the clinical severity in patients with severe FIX-deficiency (haemophilia B, HB) might be more pronounced than in those with severe HA. In this context, it is also noteworthy that thrombin generation *in vitro* in FIXa-deficient plasmas with undetectable FIX:C (the lowest limit of detection in our laboratory is <0.2 IU/dl (18)) was significantly lower than in severe HA (unpublished observation). It is well known, however, that the clinical symptoms in severe HA are more marked than in severe HB (40, 41). The reasons for these findings remain unclear, but it may be that additional mechanism(s) underlie the AHA phenotype. For example, FX may be sequestered in a non-functional complex with FVIII-anti-C2 autoAbs in AHA. Further investigations are required to clarify these mechanisms.

The current investigations have introduced a putative mechanism for the excessive clinical haemorrhagic state in AHA, although further studies are required to support this conclusion, and to clarify the clinical differences between different types of AHA. Nevertheless, treatment of AHA in patients with high titre inhibitors has historically involved the use of coagulation-bypassing agents. Meeks et al. (9) suggested, however, that administration of high-doses of FVIII should be considered more actively for patients with AHA anti-C2 type 2 inhibitors, but not in those with type 1 inhibitors. Their conclusion was based on the findings that the activity of high-titer type 2 inhibitors could be neutralised by increasing dosages of FVIII, and it may be that in the presence of low concentrations of exogenous FVIII, anti-C2 IgGs, complexed with FVIII, indirectly disturb the association between FIXa and FX. At high doses of exogenous FVIII, inhibitory activity could be completely neutralised, and unbound (free) FVIII would be available to participate in tenase assembly. Type 1 patients failed to respond to high-dose FVIII, however, and our present data are not totally consistent with those findings. The challenging observations warrant further investigation.

Acknowledgement

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Conflict of interest

K.N. has received a grant from Bayer Haemophilia Award 2009.

References

1. Mann KG, Nesheim ME, Church WR, et al. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood 1990; 76: 1–16.
2. Hoyer LW. The factor VIII complex: structure and function. Blood 1981; 58: 1–13.
3. Wood WI, Capon DJ, Simonsen CC, et al. Expression of active human factor VIII from recombinant DNA clones. Nature 1984; 312: 330–337.
4. Eaton D, Rodriguez H, Vehar GA. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. Biochemistry 1986; 25: 505–512.
5. Fay PJ. Activation of factor VIII and mechanisms of cofactor action. Blood Rev 2004; 18: 1–15.
6. Nogami K, Shima M, Matsumoto T, et al. Mechanisms of plasmin-catalyzed inactivation of factor VIII: a crucial role for proteolytic cleavage at Arg336 responsible for plasmin-catalyzed factor VIII inactivation. J Biol Chem 2007; 282: 5287–5295.
7. Gawryl MS, Hoyer LW. Inactivation of factor VIII coagulant activity by two different types of human antibodies. Blood 1982; 60: 1103–1109.

8. Prescott R, Nakai H, Saenko EL, et al. The inhibitor antibody response is more complex in hemophilia A patients than in most nonhemophiliacs with factor VIII autoantibodies. Recombinate and Kogenate Study Groups. Blood 1997; 89: 3663–3671.
9. Meeks SL, Healey JF, Parker ET, et al. Antihuman factor VIII C2 domain antibodies in hemophilia A mice recognize a functionally complex continuous spectrum of epitopes dominated by inhibitors of factor VIII activation. Blood 2007; 110: 4234–4242.
10. Meeks SL, Healey JF, Parker ET, et al. Nonclassical anti-C2 domain antibodies are present in patients with factor VIII inhibitors. Blood 2008; 112: 1151–1153.
11. Andersson LO, Brown JE. Interaction of factor VIII-von Willebrand factor with phospholipid vesicles. Biochem J 1981; 200: 161–167.
12. Scandella D, Gilbert GE, Shima M, et al. Some factor VIII inhibitor antibodies recognize a common epitope corresponding to C2 domain amino acids 2248 through 2312, which overlap a phospholipid-binding site. Blood 1995; 86: 1811–1819.
13. Shima M, Nakai H, Scandella D, et al. Common inhibitory effects of human anti-C2 domain inhibitor alloantibodies on factor VIII binding to von Willebrand factor. Br J Haematol 1995; 91: 714–721.
14. Nogami K, Shima M, Nishiya K, et al. Human factor VIII inhibitor alloantibodies with a C2 epitope inhibit factor Xa-catalyzed factor VIII activation: a new anti-factor VIII inhibitory mechanism. Thromb Haemost 2002; 87: 459–465.
15. Hoffmann M, Monroe DM. A cell-based model of hemostasis. Thromb Haemost 2001; 85: 958–965.
16. Hemker HC, Giesen P, Al Dieri R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb 2003; 33: 4–15.
17. Braun PJ, Givens TB, Stead AG, et al. Properties of optical data from activated partial thromboplastin time and prothrombin time assays. Thromb Haemost 1997; 78: 1079–1087.
18. Matsumoto T, Shima M, Takeyama M, et al. The measurement of low levels of factor VIII or factor IX in hemophilia A and hemophilia B plasma by clot waveform analysis and thrombin generation assay. J Thromb Haemost 2006; 4: 377–384.
19. Shima M, Matsumoto T, Ogiwara K. New assays for monitoring haemophilia treatment. Haemophilia 2008; 14 (Suppl 3): 83–92.
20. Matsumoto T, Nogami K, Ogiwara K, et al. A modified thrombin generation test for investigating very low levels of factor VIII activity in hemophilia A. Int J Hematol 2009; 90: 576–582.
21. Collins PW, Hirsch S, Baglin TP, et al. Acquired hemophilia A in the United Kingdom: a 2-year national surveillance study by the United Kingdom Haemophilia Centre Doctors' Organisation. Blood 2007; 109: 1870–1877.
22. Griffin BD, Micklem LR, McCann MC, et al. The production and characterisation of a panel of ten murine monoclonal antibodies to human procoagulant factor VIII. Thromb Haemost 1986; 55: 40–46.
23. Yoshioka A, Giddings JC, Thomas JE, et al. Immunoassays of factor IX antigen using monoclonal antibodies. Br J Haematol 1985; 59: 265–275.
24. Mimms LT, Zampighi G, Nozaki Y, et al. Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. Biochemistry 1981; 20: 833–840.
25. Nogami K, Wakabayashi H, Schmidt K, et al. Altered interactions between the A1 and A2 subunits of factor VIIIa following cleavage of A1 subunit by factor Xa. J Biol Chem 2003; 278: 1634–1641.
26. Pipe SW, Kaufman RJ. Characterization of a genetically engineered inactivation-resistant coagulation factor VIIIa. Proc Natl Acad Sci USA 1997; 94: 11851–11856.
27. Takeshima K, Smith C, Tait J, et al. The preparation and phospholipid binding property of the C2 domain of human factor VIII. Thromb Haemost 2003; 89: 788–794.
28. Shima M, Scandella D, Yoshioka A, et al. A factor VIII neutralizing monoclonal antibody and a human inhibitor alloantibody recognizing epitopes in the C2 domain inhibit factor VIII binding to von Willebrand factor and to phosphatidylserine. Thromb Haemost 1993; 69: 240–246.
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–254.
30. Kasper CK, Aledort LM, Aronson D, et al. A more uniform measurement of factor VIII inhibitors. Thromb Diath Haemorrh 1975; 34: 869–872.
31. Suzuki H, Shima M, Arai M, et al. Factor VIII Ise (R2159C) in a patient with mild hemophilia A, an abnormal factor VIII with retention of function but modification of C2 epitopes. Thromb Haemost 1997; 77: 862–867.
32. Lollar P, Fay PJ, Fass DN. Factor VIII and factor VIIIa. Methods Enzymol 1993; 222: 128–143.
33. Saenko EL, Shima M, Rajalakshmi KJ, et al. A role for the C2 domain of factor VIII in binding to von Willebrand factor. J Biol Chem 1994; 269: 11601–11605.
34. Nogami K, Shima M, Hosokawa K, et al. Factor VIII C2 domain contains the thrombin-binding site responsible for thrombin-catalyzed cleavage at Arg1689. J Biol Chem 2000; 275: 25774–25780.
35. Saenko EL, Shima M, Gilbert GE, et al. Slowed release of thrombin-cleaved factor VIII from von Willebrand factor by a monoclonal and a human antibody is a novel mechanism for factor VIII inhibition. J Biol Chem 1996; 271: 27424–27431.
36. 36.Ngo JC, Huang M, Roth DA, et al. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. Structure 2008; 16: 597–606.
37. Nogami K, Saenko EL, Takeyama M, et al. Identification of a thrombin-interactive site within the FVIII A2 domain that is responsible for the cleavage at Arg372. Br J Haematol 2008; 140: 433–443.
38. Soeda T, Nogami K, Nishiya K, et al. The factor VIIIa C2 domain (residues 2228–2240) interacts with the factor IXa Gla domain in the factor Xase complex. J Biol Chem 2009; 284: 3379–3388.
39. Lollar P, Hill-Eubanks DC, Parker CG. Association of the factor VIII light chain with von Willebrand factor. J Biol Chem 1988; 263: 10451–10455.
40. Schulman S, Eelde A, Holmström M, et al. Validation of a composite score for clinical severity of hemophilia. J Thromb Haemost 2008; 6: 1113–1121.
41. Tagariello G, Iorio A, Santagostino E, et al. Comparison of the rates of joint arthroplasty in patients with severe factor VIII and IX deficiency: an index of different clinical severity of the 2 coagulation disorders. Blood 2009; 114: 779–784.

Assessment of F/HN-Pseudotyped Lentivirus as a Clinically Relevant Vector for Lung Gene Therapy

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Rationale: **Ongoing efforts to improve pulmonary gene transfer thereby enabling gene therapy for the treatment of lung diseases, such as cystic fibrosis (CF), has led to the assessment of a lentiviral vector (simian immunodeficiency virus [SIV]) pseudotyped with the Sendai virus envelope proteins F and HN.**

Objectives: **To place this vector onto a translational pathway to the clinic by addressing some key milestones that have to be achieved.**

Methods: **F/HN-SIV transduction efficiency, duration of expression, and toxicity were assessed in mice. In addition, F/HN-SIV was assessed in differentiated human air–liquid interface cultures, primary human nasal epithelial cells, and human and sheep lung slices.**

Measurements and Main Results: **A single dose produces lung expression for the lifetime of the mouse (~2 yr). Only brief contact time is needed to achieve transduction. Repeated daily administration leads to a dose-related increase in gene expression. Repeated monthly administration to mouse lower airways is feasible without loss of gene expression. There is no evidence of chronic toxicity during a 2-year study period. F/HN-SIV leads to persistent gene expression in human differentiated airway cultures and human lung slices and transduces freshly obtained primary human airway epithelial cells.**

Conclusions: **The data support F/HN-pseudotyped SIV as a promising vector for pulmonary gene therapy for several diseases including CF. We are now undertaking the necessary refinements to progress this vector into clinical trials.**

Keywords: lentivirus; cystic fibrosis; gene therapy; lung; gene transfer

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

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 cystic fibrosis (CF), because of immunogenicity, which prevents successful repeat administration. Lentivirus-based vectors are a notable exception.

What This Study Adds to the Field

Moving novel therapies to the clinic requires that relevant evidence for safety and efficacy is gathered in appropriate models. Here, we provide a body of supportive evidence for F/HN-pseudotyped simian immunodeficiency virus as a potential gene transfer agent for CF including lifetime gene expression and efficient repeat administration in mouse lung, lack of chronic toxicity, and persistent gene expression in human *ex vivo* models.

Gene transfer to the airway epithelium has been more difficult than originally anticipated, largely because of significant extracellular and intracellular barriers in the lung (1). In general, viral vectors are more adapted to overcoming these barriers than nonviral gene transfer agents. Viral vectors that have a natural tropism for the airway epithelium, such as those derived from adenovirus, adeno-associated virus, and Sendai virus (SeV), have been evaluated for cystic fibrosis (CF) gene therapy. SeV vector, in particular, leads to log orders higher gene expression than nonviral formulation when applied to the apical surface of airway epithelial cells (2, 3). However, gene expression is transient and repeated administration is inefficient (4, 5). These vectors are, therefore, unlikely to solve the challenge of life-long gene therapy treatment for CF.

Lentiviral vectors are commonly pseudotyped with the G-glycoprotein from the vesicular stomatitis virus (VSV-G) allowing for a broad tissue tropism. However, VSV-G–pseudotyped vectors are comparatively inefficient at transducing airway epithelial cells and require the addition of tight junction openers, such as lysophosphatidylcholine, to allow virus entry into airway cells (6, 7). Several groups have attempted to further improve lentiviral vector uptake into airway epithelium by changing the viral envelope proteins. Glycoproteins from Ebola or Marburg virus that naturally transfect airway epithelial cells by the apical membrane showed early promise (8), but have more recently been superseded by viral vectors pseudotyped with the influenza M2 envelope glycoprotein (9), baculovirus protein GP64 (10), or the SeV-derived F and HN envelope proteins (11, 12).

The F/HN-pseudotyped simian immunodeficiency viral vector (F/HN-SIV) transduces rodent airway epithelial cells *in vitro* (12). Recently, we have shown that F/HN-SIV leads to persistent expression in the mouse nose (>1 yr) importantly allowing for monthly repeat administration without significant loss of efficacy (11). It is currently unclear whether the prolonged expression is caused by vector integration into pulmonary stem or progenitor cells, or by the long life-span of airway epithelial cells, which as recently reported may have a half-life of up to 17 months (13). It is also unclear how on repeated administration

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the viral vector evades the immune system, although interestingly liposomes that contain SeV proteins (HVJ-liposome) can also be repeatedly administered (14). This feature, and the efficient and prolonged expression profile and the ability to administer through the apical surface of the respiratory epithelium without preconditioning, makes the vector an attractive candidate for treating CF, which is a chronic disease and requires lifelong correction of the genetic defect in airway epithelial cells.

Here, we have further developed the F and HN–pseudotyped SIV toward clinical evaluation. We show that repeated administration to the mouse lower airways is feasible. We also confirm the long-term safety profile of this viral vector and show that it transduces the relevant human airway epithelial cells required for CF gene therapy.

METHODS

Viral Vector Production

Purified and concentrated F/HN-SIV expressing firefly luciferase (F/HN-SIV-Lux) or enhanced green fluorescent protein (F/HN-SIV-GFP) under the transcriptional control of the cytomegalovirus (CMV) enhancer/promoter were prepared and titrated as previously described (11) (*see* online supplement for further details).

Mouse Lung Transfection

Female C57BL/6N mice (6–8 wk old) were used (*see* online supplement). A 100-μl viral vector in Dulbecco's phosphate-buffered saline (D-PBS) was administered to the mouse nose and "sniffed" into the lung as previously described (15) (*see* RESULTS and FIGURES for details about vector titers used) and gene expression quantified (*see* online supplement).

For the daily repeat administration experiments, groups of mice were treated over 10 days with either nine daily doses of D-PBS followed by a single dose of F/HN-SIV-Lux, five daily doses of D-PBS followed by five daily doses of F/HN-SIV-Lux, or 10 daily doses of F/HN-SIV-Lux. Gene expression was analyzed 28 days after the final F/HN-SIV-Lux administration.

For the monthly repeat administration experiments groups of mice were transduced with either 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). Importantly, mice receiving F/HN-SIV-Lux (single-dose group) and F/HN-SIV-Lux on Day 56 (repeat-dose group) were of similar age and were transduced at the same time. Gene expression was analyzed 28 days after F/HN-SIV-Lux administration.

Toxicology

Over the 24-month study period mice were carefully observed daily and were given a full clinical examination every 2 weeks (including palpation of the abdomen for tumors) by an experienced animal technician, and bodyweight was recorded. Histologic assessment was performed in mice that showed signs of illness throughout the study period. In addition, lungs from asymptomatic mice culled at the end of the 24-month study period were also analyzed (*see* online supplement).

Histologic Assessment of GFP Expression

Mouse lungs were transduced with F/HN-SIV-GFP (10^8 TU/mouse in 100 μl) by nasal sniffing. Animals receiving D-PBS only were used as negative controls (n = 4 per group). One month after transduction animals were culled and GFP expression was assessed in lung tissue (*see* online supplement).

Gene Transfer into Relevant Preclinical Model

Gene transfer into human air–liquid interface (ALI) cultures, human nasal brushings, and lung slices was performed as described in the online supplement.

Statistical Analysis

Analysis of variance followed by a Bonferroni *post hoc* test or Kruskal-Wallis test followed by Dunn multiple comparison *post hoc* test was performed for multiple group comparison after assessing parametric and nonparametric data distribution with the Kolmogorov-Smirnov normality test, respectively. An independent Student *t* test or a Mann-Whitney test was performed for two-group parametric and nonparametric data as appropriate. Pearson correlation was performed for parametric data. All analyses were performed using GraphPad Prism4 (GraphPad Software, Inc., La Jolla, CA) and the null hypothesis was rejected at P less than 0.05.

RESULTS

Gene Expression in Mouse Lung Persists and Is Stable

We first assessed if F/HN-SIV produced measurable levels of luciferase in murine lower airways (lungs), and if this expression was dose-related. Mice were transduced with F/HN-SIV-Lux (10^7 or 10^8 TU/mouse in 100 μl total volume) by nasal sniffing (n = 8 per group) or received D-PBS (n = 8). Two days after transduction mice were culled and luciferase expression quantified in lung homogenates. Luciferase expression was detectable and dose-related (10^7 TU/mouse, 2 ± 0.3 relative light units [RLU])/mg protein; 10^8 TU/mouse, 27 ± 6.4 RLU/mg protein, $P < 0.01$; D-PBS, 0.01 ± 0.03 RLU/mg). However, gene expression was also production batch-related (*see* online supplement).

We next assessed if expression in mouse lungs persisted. Mice were transduced with F/HN-SIV-Lux (5×10^8 TU/mouse in 100 μl total volume) by nasal sniffing (n = 8 in two independent experiments) or received D-PBS (negative controls, n = 6) and luciferase expression was quantified using *in vivo* bioluminescence imaging (BLI) at regular intervals for up to 22 months. Luciferase-mediated photon emission was detectable in all treated mice 2 months after transduction (SIV, 362,660 ± 63,922 photons/s/cm^2, n = 8; D-PBS, 66,535 ± 4,868 photons/s/cm^2, n = 6; $P < 0.005$). Seven out of eight mice survived for 16 months and four out of the eight mice survived until termination of the experiment at 22 months. All of the SIV transduced mice had detectable luciferase expression at all time points. Photon emission increased modestly, but significantly ($P < 0.005$), over time (SIV at 2 mo, 381,123 ± 70,665 photons/s/cm^2; at 16 months, 543,156 ± 65,234 photons/s/cm^2; n = 7 mice with data for both time-points). This was maintained at 22 months (2 mo, 367,485 ± 115,923 photons/s/cm^2; 22 months, 1,407,000 ± 435,790 photons/s/cm^2; n = 4 mice with data for both time-points). In contrast, photon emission in D-PBS–treated mice remained stable over the same time period (Figures 1A and 1B).

An increase in photon emission may indicate an increase in luciferase expression potentially caused by an increase in the number of luciferase-expressing cells over time or may be a peculiarity of BLI. We therefore repeated the experiment comparing BLI with post-mortem quantification of luciferase expression in lung tissue homogenates in the same animals. Mice were transduced with F/HN-SIV (10^7 TU/mouse, n = 8–12 per time-point) and at regular intervals over a 24-month period (for technical reasons BLI could only be performed between 6 and 24 months after gene transfer) and post-mortem quantification of luciferase expression in lung tissue homogenate was performed in cohorts of mice. Similar to results described previously photon emission significantly ($P < 0.001$) increased over the study period (BLI at 6 mo, 87,915 ± 8,871 photons/s/cm^2; BLI at 24 mo, 310,334 ± 36,274 photons/s/cm^2) (Figure 1C), whereas detectable levels of luciferase in tissue homogenate remained stable (Figure 1D). There was no correlation between *in vivo* photon emission and luciferase expression quantified in tissue homogenates (Spearman $r = 0.205$; $P = 0.11$; n = 63 paired data points from 6–24 mo). However, luciferase expression quantified in tissue homogenates correlated well with photon emission from the same animal when lungs were extracted before BLI (Figure 1E) (Spearman $r = 0.75$; $P = 0.01$; n = 11 paired data points at 24 mo), which implies that photon quenching may occur *in vivo*.

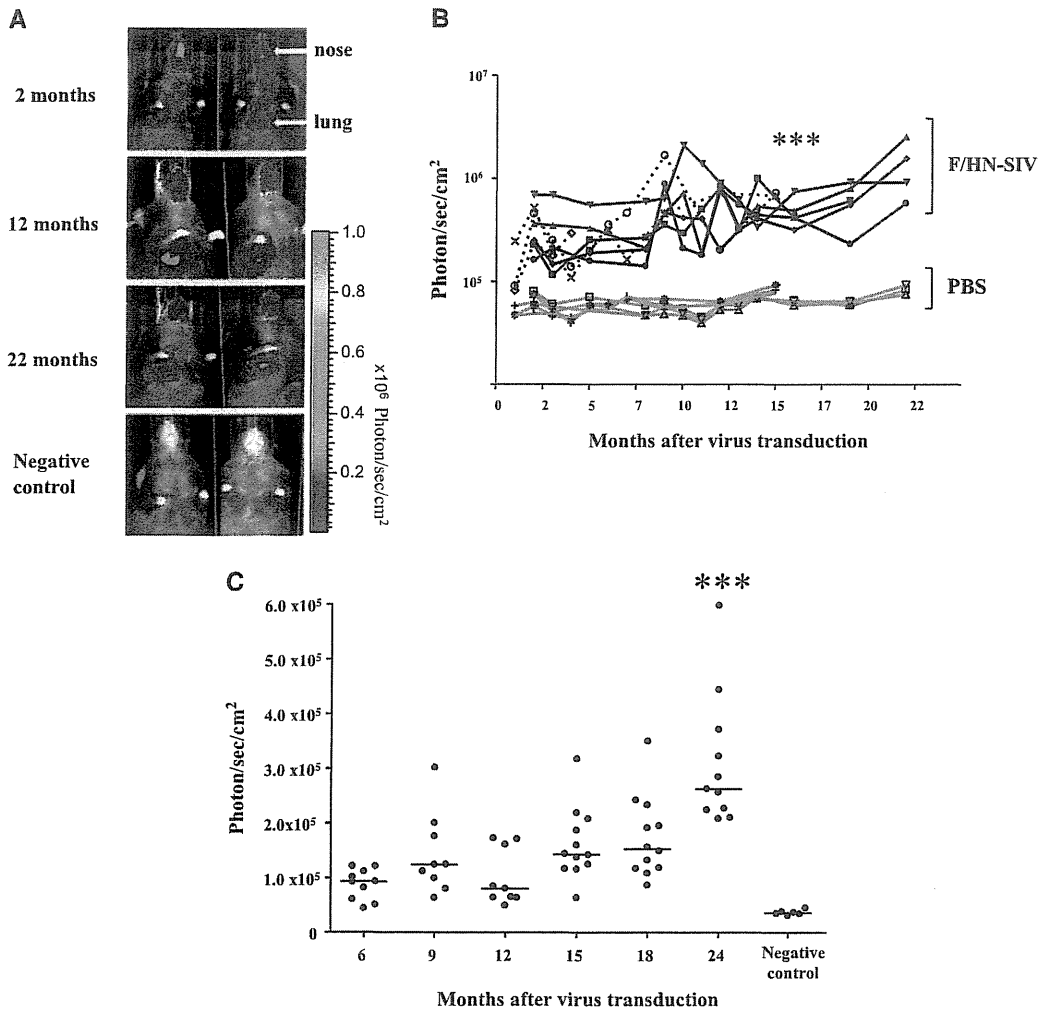


Figure 1. F/HN simian immunodeficiency virus (SIV) transduction leads to persistent gene expression in mouse lung. Mice were transduced with F/HN-SIV-luciferase (Lux) (5×10^8 TU/mouse) by nasal sniffing (n = 8 in two independent experiments) or received phosphate-buffered saline (PBS) (negative controls, n = 6). Lux expression was quantified using bioluminescence imaging (BLI) or in tissue homogenate at regular intervals for up to 24 months. (*A*) BLI 2–22 months after transduction. Representative images of two mice reaching the 22 months time point are shown. (*B*) Quantification of *in vivo* BLI over time (*black lines*, n = 8) or PBS (*red lines*, n = 6). Each line represents photon emission over time in one animal. *Solid* and *dotted lines* represent independent experiments. ***$P < 0.005$ when compared with Month 2. BLI (*C*) and Lux expression in lung tissue homogenate (*D*) was repeated at regular intervals over 24 months in a third independent experiment (10^7 TU/mouse, = 8–12 per timepoint). For technical reasons BLI could only be performed between 6 and 24 months after gene transfer. ***$P <$ 0.005 when compared with Month 6. Quantification of Lux expression in lung tissue homogenate was performed at regular intervals over the 24-month study period. Each *dot* represents one animal. *Horizontal bars* represent the group median. (*E*) Correlation between Lux expression quantified in tissue homogenates and photons emitted from extracted lungs (representative images from 11 extracted lungs are shown). Each *dot* represents one animal. (*F*) Detection of green fluorescent protein (GFP) expression in lung after transduction with F/HN-SIV-GFP (10^8 TU/mouse). Transduced GFP-positive cells were identified using fluorescent microscopy (original magnification, ×20). (*i*) PBS-treated negative control. (*ii* and *iii*) Tissue sections from F/HN-SIV-GFP–treated mice showing GFP-positive cells in airways (*arrows*) and alveoli. Representative images from four mice per group are shown. RLU = relative light units.

Additional technical considerations relating to limitations of BLI and virus batch-to-batch variability became apparent in this study (*see* online supplement).

To determine what cell types were transduced in the lung we transfected mice with F/HN-SIV-GFP (10^8 TU/mouse) or D-PBS (n = 4 per group) by nasal sniffing and assessed GFP expression 1 month after transduction. GFP expression was detectable in airway epithelial cells, and also in the alveolar region (Figure 1F).

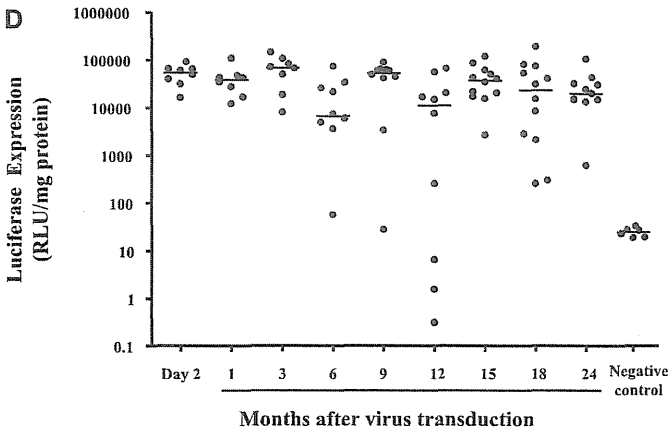
F/HN-SIV Requires Only Short Contact Time to Achieve Efficient Transduction

We compared prolonged contact time of the vector with the nasal epithelium (by perfusion) with very brief contact time (sniffing). The latter led to equally efficient transduction of the nasal epithelium as the former (Figures 2A and 2B). This suggests that a short contact time between the viral vector and the target cell may be sufficient for efficient vector uptake into the cells, of potential importance in future clinical trials.

Gene expression in the nose also persisted for 15–22 months in seven out of eight mice, but in contrast to the lung gradually declined by approximately 60% over this period (SIV Month 2, 1,309,000 ± 316,612 photons/s/cm^2; SIV Month 15, 583,951 ± 228,804 photons/s/cm^2, $P < 0.05$, n = 7 per group; D-PBS Month 15, 53,021 ± 2,325 photons/s/cm^2, $P < 0.005$), which may be caused by different cell types being transduced in nose and lung or different turnover rates of the cells at these two sites. Consistent with our previous data using nonviral gene transfer agents (16), intranasal administration of luciferin (the substrate for luciferase) boosted F/HN-SIV-Lux–derived photon emission in mice that were negative after intraperitoneal administration of luciferin (Figure 2C).

Daily Repeat Administration to the Lung Is Feasible

Although gene expression after a single dose of F/HN-SIV persists for the lifetime of the animal, a single dose may not be sufficient to achieve therapeutic benefit in humans. It is, for example, conceivable that the total volume required for delivery of the optimal vector titer to the human lung may be too large for a single dose and administration may have to be split into several doses to accommodate the volume. We, therefore, assessed if repeated daily



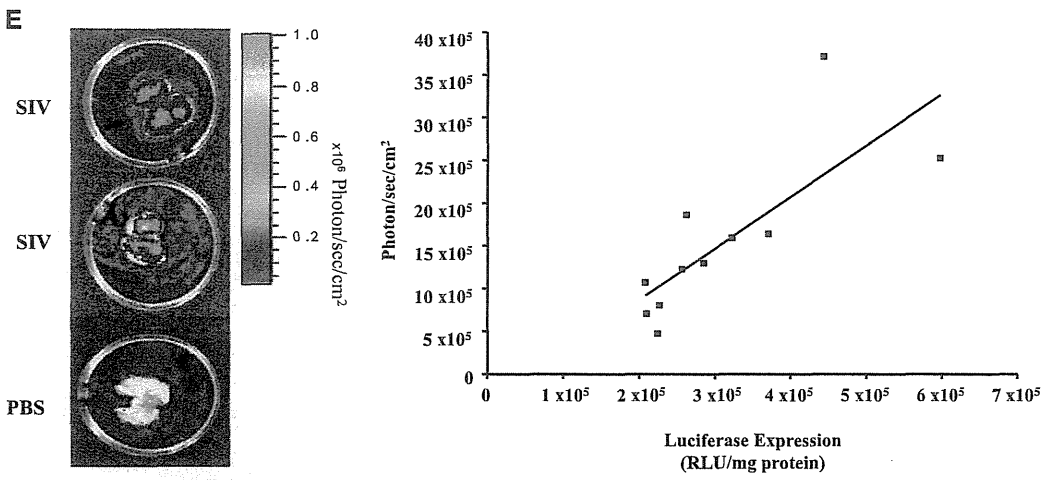
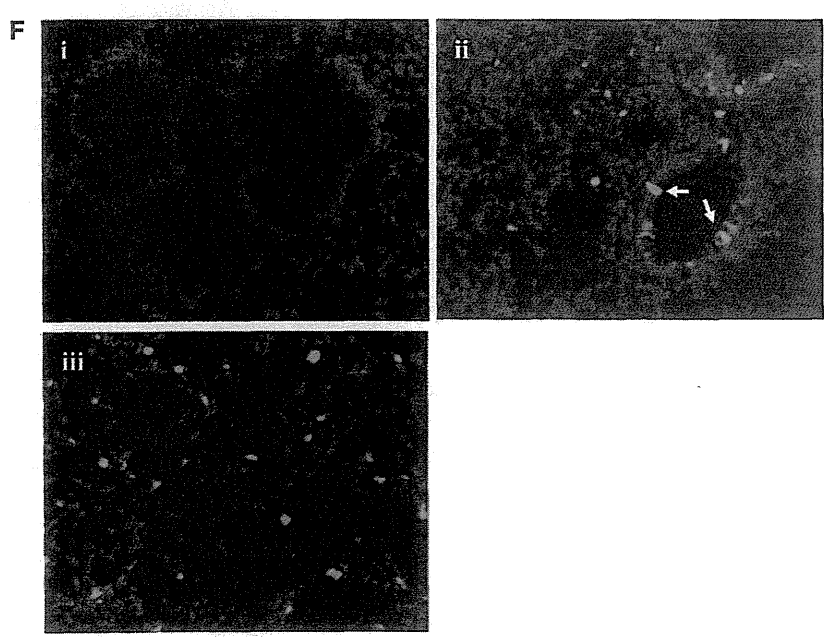


Figure 1. (*Continued*).



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

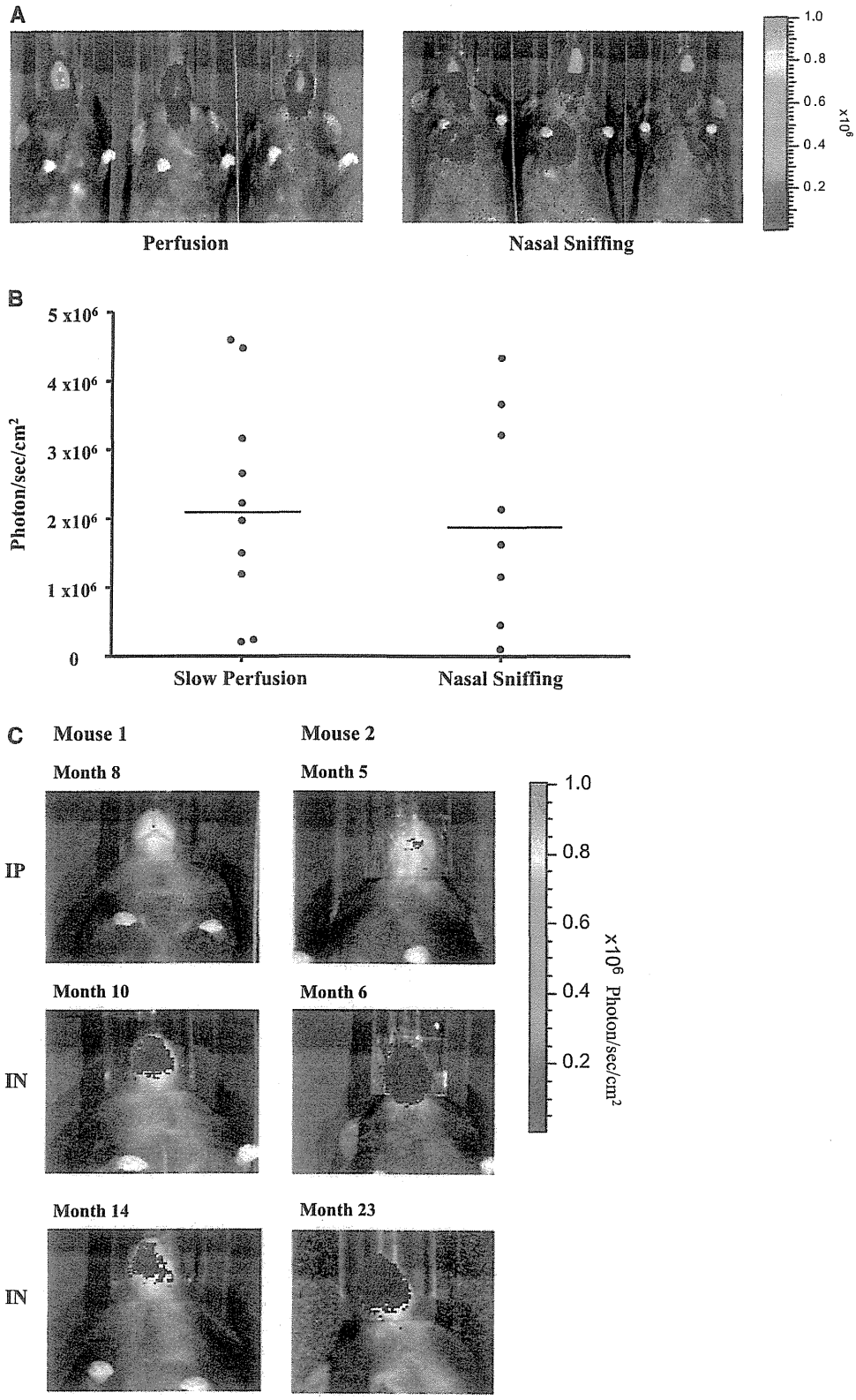


Figure 2. F/HN simian immunodeficiency virus (SIV) requires only short contact time to achieve efficient transduction. Luciferase (Lux) expression was compared in mice receiving F/HN-SIV-Lux ($3–4 \times 10^8$ TU/mouse in 100 μl) by slow perfusion (1.3 μl/min, n = 10) or as bolus administration by nasal 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.

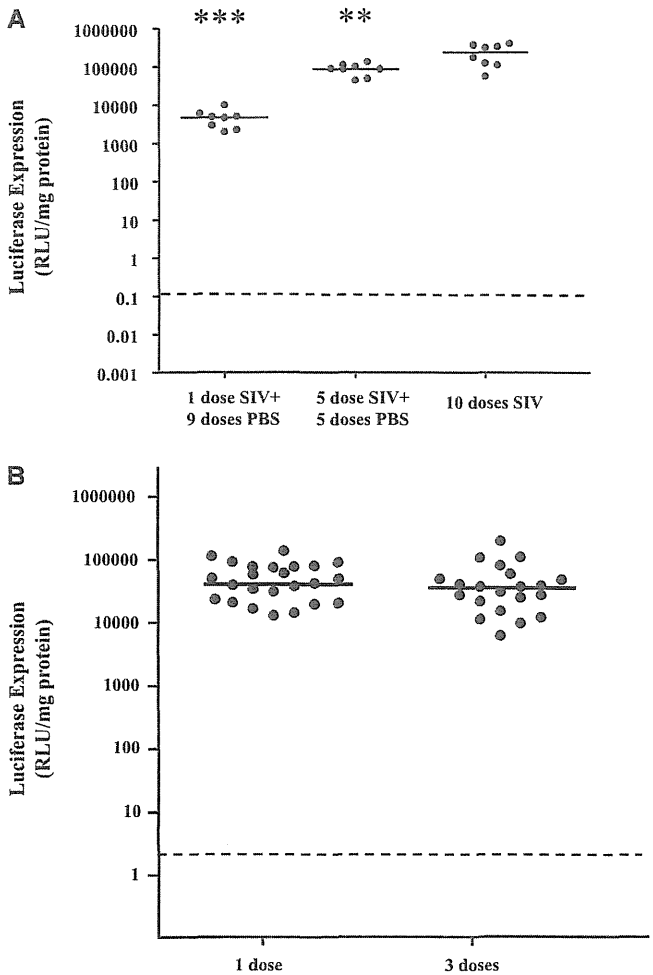


Figure 3. Daily and monthly repeat administration to the lung is feasible. (*A*) Daily readministration. Mice (n = 8 per group) were treated with either F/HN simian immunodeficiency virus (SIV) luciferase (Lux) for 10 days (10^6 TU/day) or with D phosphate buffered saline (PBS) for 5 days followed by 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

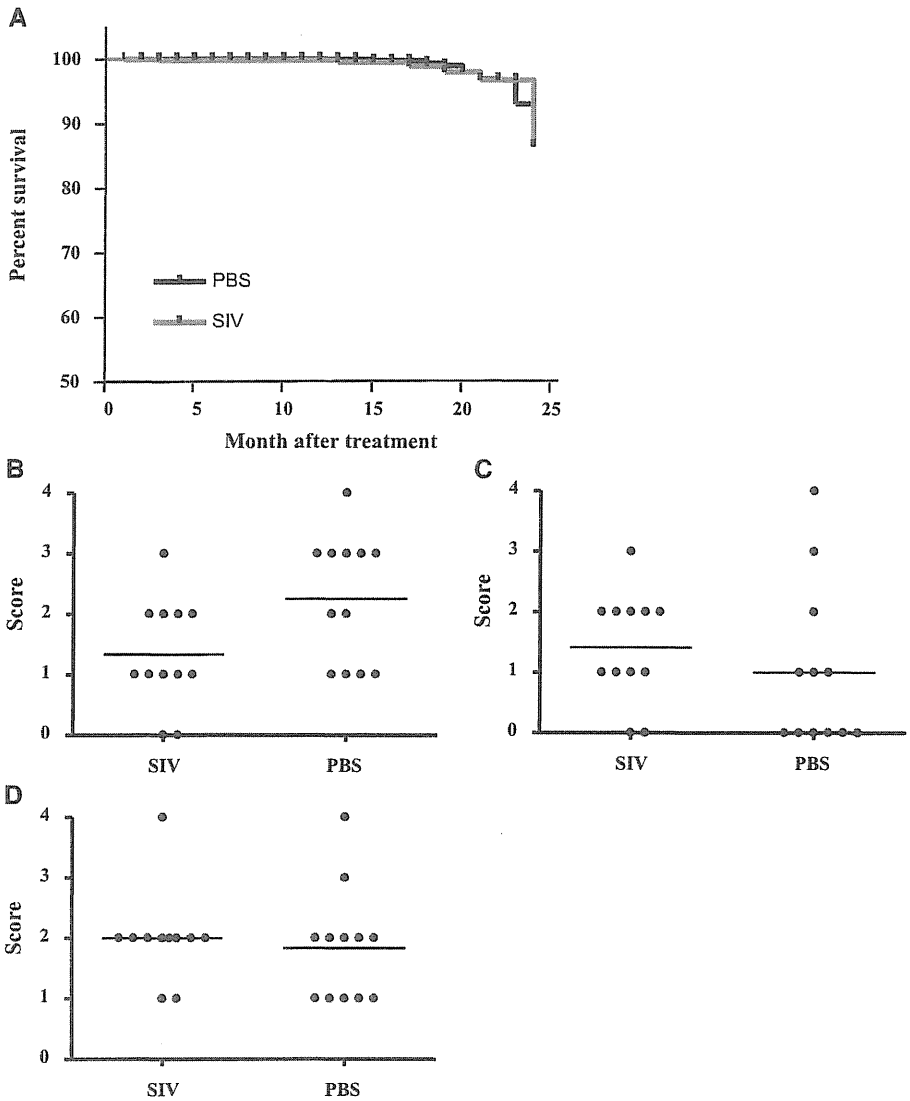


Figure 4. No evidence of chronic toxicity during 2-year follow-up period. (*A*) Twenty-four months survival of mice treated with F/HN simian immunodeficiency virus (SIV) luciferase (Lux) (10^7 TU/mouse, n = 99 at start of the experiment) or phosphate-buffered saline (PBS) (n = 48 at start of the experiment) 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

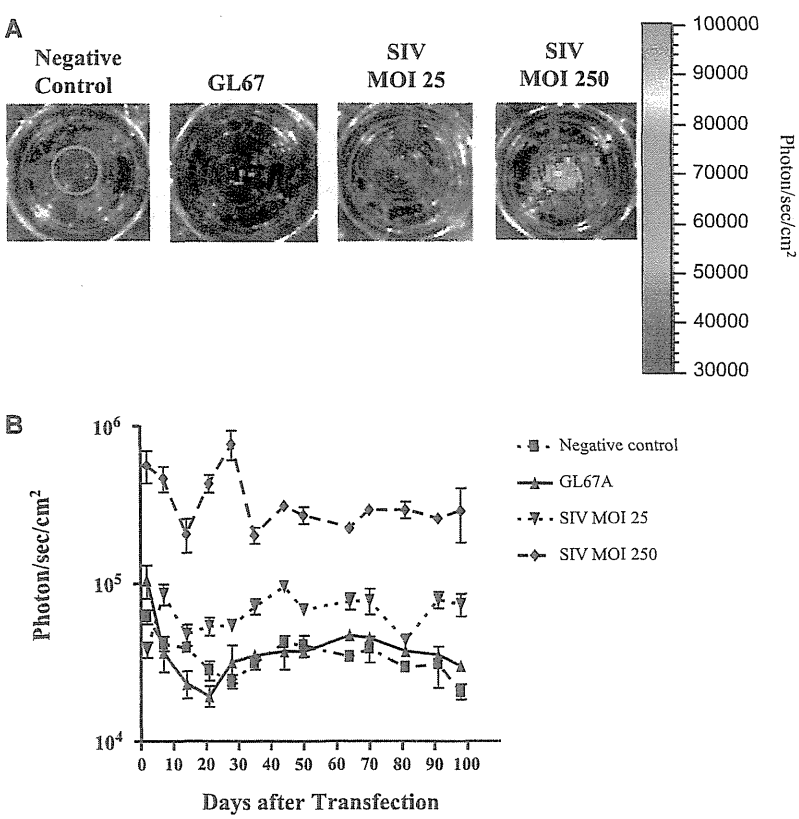


Figure 5. Persistent gene expression in differentiated air–liquid interface (ALI) cultures. Differentiated human ALI cultures were transduced with F/HN simian immunodeficiency virus (SIV) luciferase (Lux) at multiplicity of infection (MOI) 25 or 250 (n = 5 per group), transfected with GL67A complexed 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.

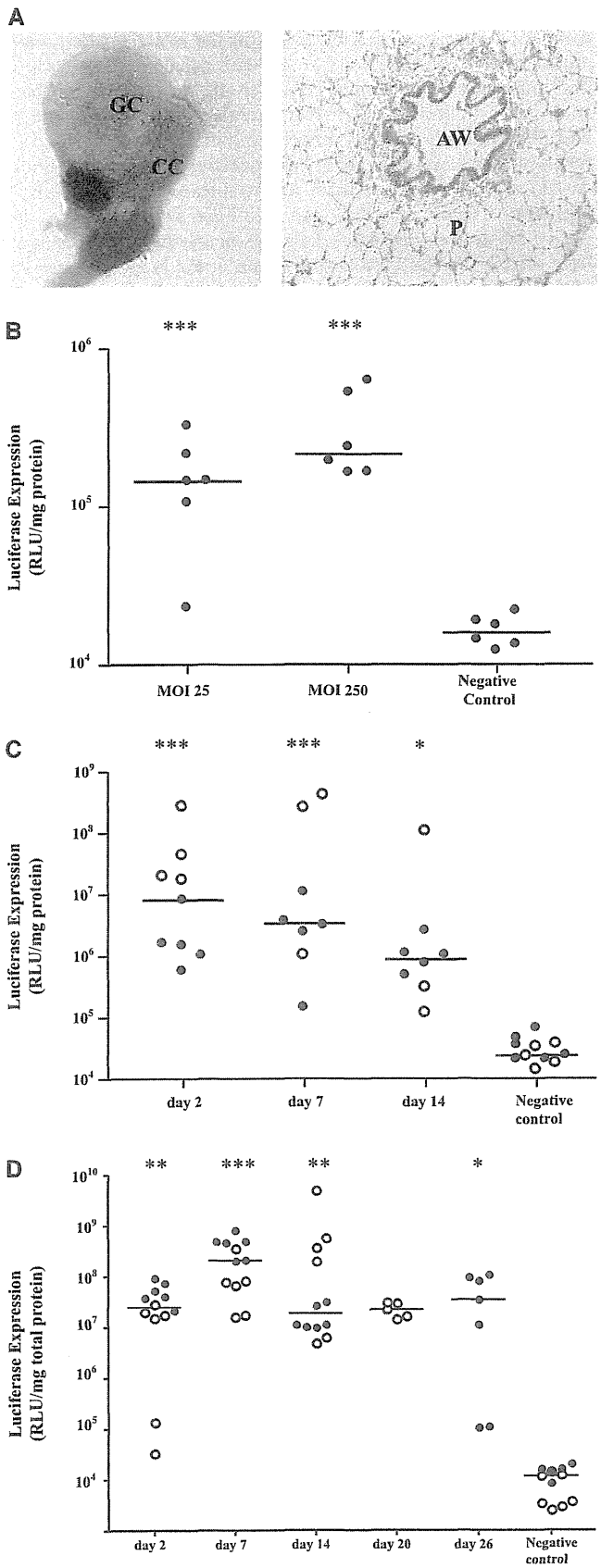


Figure 6. F/HN simian immunodeficiency virus (SIV) transduces primary human pulmonary cells. (*A*) Microscopic section showing primary human airway epithelial cells obtained from nasal brushings (*left*, GC = goblet cell, CC = ciliated airway epithelial cell) or human lung slices (*right*, AW = airway, 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

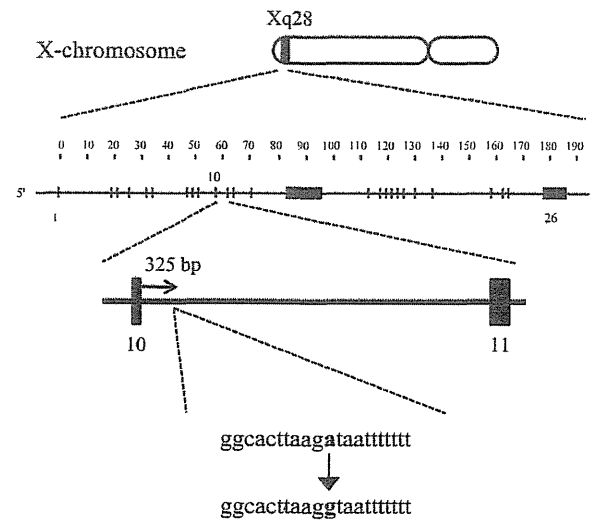

Fig. 1. Adenine to guanine transition in intron 10 of *F8*. The adenine to guanine transition was detected 325 bp downstream from the 3′ end of exon 10 (c.1478 + 325A>G).

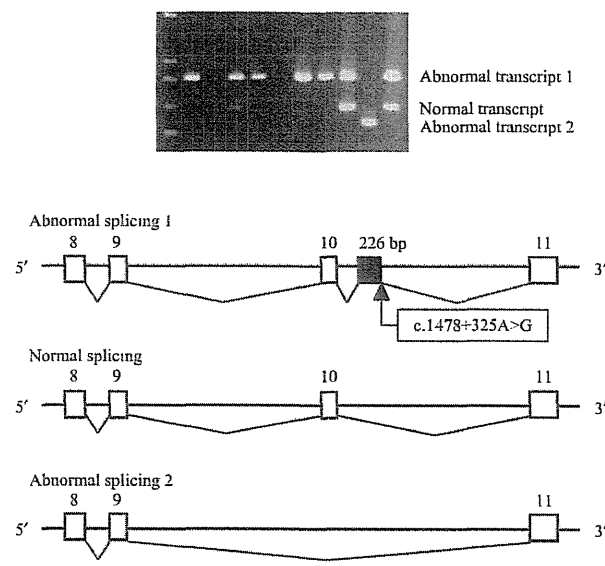

Fig. 2. *F8* mRNA splicing in the patient. The transcript of the patient was analysed by nested RT-PCR. After the amplification of exon 8–14 by RT-PCR, exon 8–11 was then amplified by using nested primers. Two abnormal sized transcripts (abnormal transcript 1 and 2), were detected by 10 independently 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.

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

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

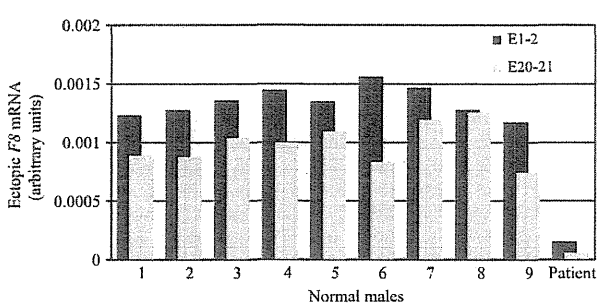

Fig. 3. Ectopic *F8* mRNA quantification. *F8* mRNA levels were relatively quantified by using two different regions, upstream of the transition (exon 1–2) and downstream of the transition (exon 20–21). The result was normalized with endogenous control β-actin and expressed relative to the *F8* level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

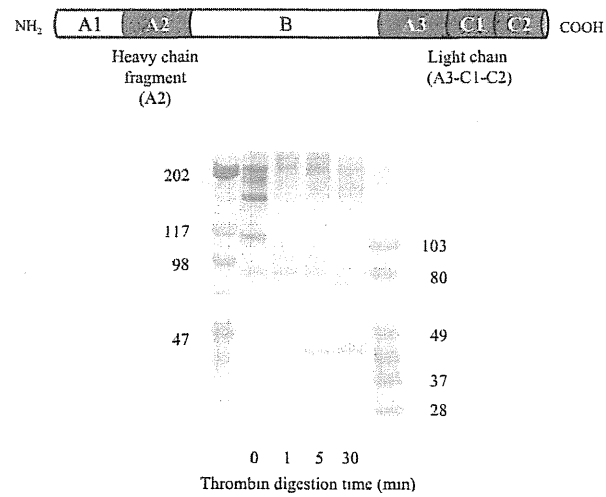

Fig. 4. Inhibitor epitope analysis. The inhibitor recognizes both the A2 domain (44 kDa) and the light chain (A3-C1-C2 domain; 83 kDa) of FVIII.

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