We also used *in vivo* bioluminescence imaging after nasal perfusion with F/HN-SIV-lux to assess gene expression over time in the same animal (Figure 3e).

Figure 3f shows quantification of photon emission after intraperitoneal injection of luciferin substrate from 1 to 8 months after transduction. Although persistence of gene expression far exceeds the expected lifespan of airway epithelial cells of 100 days, possibly indicating progenitor cell integration, repeated bioluminescence *in vivo* imaging after F/HN-SIV-lux transduction shows a gradual decline in photon emission over an 8-month period (month 1: $2.2 \times 10^6 \pm 4.9 \times 10^5$, month 8: $2.7 \times 10^5 \pm 8.8 \times 10^4$, $n = 10$/group, $P < 0.005$), but was still significantly ($P < 0.01$) higher than the PBS control ($4.5 \times 10^4 \pm 2.0 \times 10^3$, $n = 5$) This result is consistent with the decline in GFP-positive cells described above. In addition, we analyzed all data using a repeat measure test to determine whether the decline in bioluminescence stabilizes during the 8-month study period. The analysis showed that gene expression significantly ($P < 0.05$) declined for the first 4 months but then stabilized with expression levels from 5 to 8 months not being different compared to the 4-month levels.

The SIV vector–transduced cells show clustering after induced regeneration of the epithelium

To ascertain further whether F/HN-SIV induced chromosomal integration into nasal respiratory progenitor or stem cells, we artificially induced cell division after SIV vector transduction by damaging the nasal tissue with the detergent (polidocanol),[6] which has previously been shown to strip the surface epithelium within a few hours, while retaining basal cells able to regenerate the epithelium within 7 days (Figures 4a–d). At 7 and 28 days after vector transduction (4×10^8 TU/mouse, $n = 3$) the nasal tissue was perfused with 2% polidocanol (10 µl/mouse) and gene expression analyzed 4 weeks after the last detergent treatment. Importantly, GFP-expressing cells now showed clustering after polidocanol treatment (Figure 4e and Supplementary Figure S2), possibly indicating origination from a common progenitor.

SIV-mediated gene transfer can be achieved after three applications of the vector

Although the above data are encouraging, gene therapy for CF will require lifelong treatment. We therefore, assessed the feasibility of

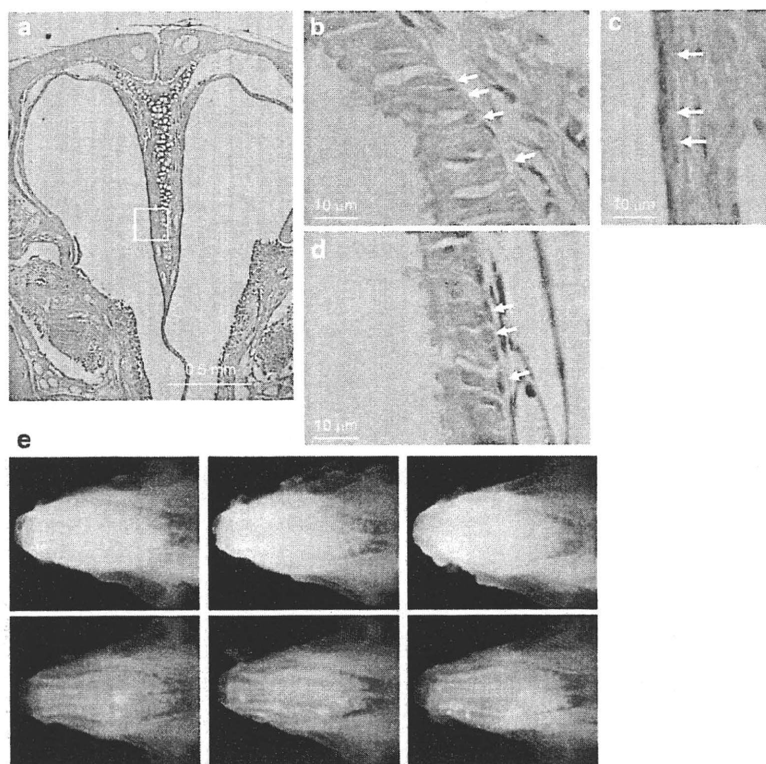

Figure 4 Clustering of transduced cells after the polidocanol-mediated stripping of epithelial cells followed by rapid regeneration. Mouse nasal tissue was perfused with 10 µl of 2% (vol/vol) polidocanol ($n = 3$). (**a**) Representative low-power view (original magnification ×50) of the nasal cavity 24 hours after perfusion. Respiratory epithelium, marked by a white box was further magnified (original magnification ×200). The respiratory epithelium before the treatment is shown in **b**. Arrow indicates basal cells. The respiratory epithelium was completely stripped 24 hours after polidocanol perfusion, whereas the basal cell layer was (**c**) retained and (**d**) regenerated 7 days after treatment. (**e**) This treatment was done after transduction with F/HN-SIV vector. Seven days after transduction of nasal epithelial cells with F/HN-SIV-GFP (4×10^8 transduction units/100 µl/mouse), the nasal epithelium was stripped via perfusion with 10 µl of 2% (vol/vol) polidocanol. Polidocanol treatment was repeated again 3 weeks later. Histological sections were analyzed 58 days after vector administration (30 days after the last polidocanol treatment). *In situ* imaging of GFP expression in the nasal cavity of untreated mice (top panel in **e**) or mice treated with polidocanol (bottom panel in **e**). Clusters of GFP-positive cells were seen in the polidocanol-treated mice. GFP, green fluorescent protein.

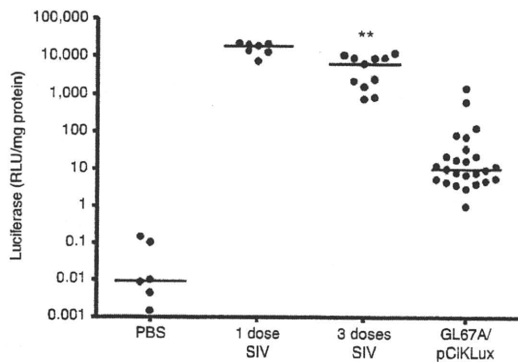

Figure 5 Repeat administration of F/HN-SIV to nasal epithelium. Mice were transduced with F/HN-SIV-lux (1 dose) or two doses of F/HN-SIV-GFP (day 0 and day 28) followed by F/HN-SIV-lux 4 weeks later (day 56 = 3 doses). Luciferase expression was measured 30 days after F/HN-SIV-lux transduction and compared to levels achieved with the non-viral gene transfer agent GL67A complexed to a luciferase reporter gene plasmid (pCIKLux). Each dot represents one mouse. Horizontal bars indicate the median per group (**$P < 0.01$) compared to mice receiving GL67A/plasmid DNA. PBS, phosphate-buffered saline; RLU, relative light units.

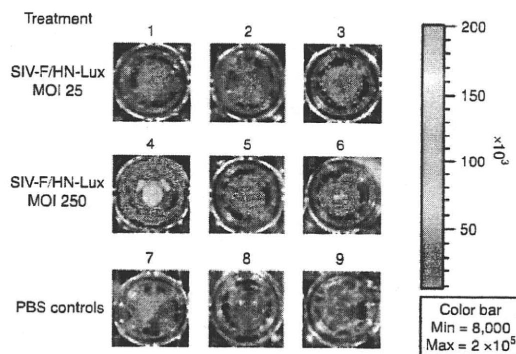

Figure 6 Transduction of human air–liquid interface (ALIs) cultures with F/HN-SIV-lux. ALIs were transduced with F/HN-SIV-lux at an approximate multiplicity of infection of 25 (1–3) and 250 (4–6) or treated with phosphate-buffered saline (PBS) (7–9). 5 days after transduction ALIs were treated with luciferin and bioluminescent imaging performed.

readministering this vector, and compared transduction efficiency to the current optimal nonviral formulation for airway gene transfer *in vivo*. **Figure 5** shows that following two administrations of F/HN-SIV-GFP separated by 1 month, a third administration of F/HN-SIV-lux (to prevent an immune response against the transgene) produced gene expression of ~40% of that seen following a single challenge with F/HN-SIV-lux. Further, these levels after three challenges with the SIV vector remained ~500-fold greater ($P < 0.01$) than seen with an optimal nonviral formulation, previously used in a CF clinical trial.

F/HN-SIV transduces differentiated human airway epithelium

Differentiated human airway epithelium is in general difficult to transduce. In preliminary experiments, we have shown that F/HN-pseudotyped lentivirus transduced human airway cells grown as air–liquid interphase (ALI) cultures. ALIs were transfected with F/HN-SIV carrying a luciferase reporter gene at

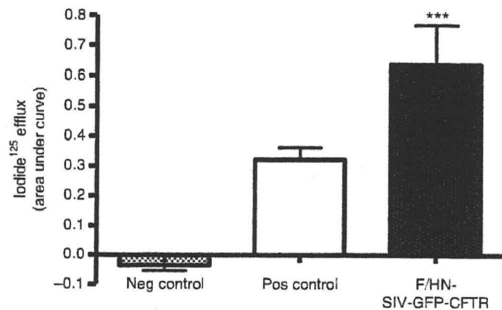

Figure 7 Functional confirmation of CF transmembrane conductance regulator (CFTR) production by F/HN-SIV-GFP-CFTR. HEK293T cells were transduced with F/HN-SIV-GFP-CFTR or a control virus carrying green fluorescent protein (GFP) (F/HN-SIV-GFP) at an multiplicity of infection of 500. The iodide efflux assay was performed 2 days after transduction. Cells transfected with an eukaryotic expression plasmid carrying the *CFTR* complementary DNA under the control of a cytomegalovirus promoter complexed to Lipofectamine 2000 were used as positive control. Data are presented as mean ± SEM. ***$P < 0.001$ compared to the control virus, $n = 6$/group. Neg, negative; pos, positive.

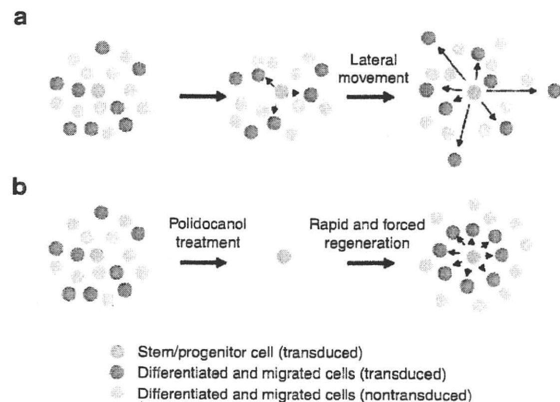

Figure 8 Schematic representation of epithelial cell migration in intact and damaged epithelium. (**a**) Scattered pattern with regeneration in normal condition. (**b**) Clustered formation after rapid and forced regeneration. We speculate that under normal physiological (undamaged) conditions, turnover may be comparatively slow and that newly generated epithelial cells may move laterally away from the stem or progenitor cell that they originated from **a**. In contrast, if rapid regeneration is forced (after tissue damage with polidocanol) stem or progenitor cells have to divide rapidly and newly generated epithelial cells may (transiently?) stay in closer proximity to the cell that they originated from **b**. This hypothesis may explain, why we observed clusters of GFP-positive cells in damaged, but not in undamaged epithelium.

an approximate multiplicity of infection of 100 and luciferase expression was detectable 10 and 26 days after gene transfer (day 10: 53.0 ± 6.3 relative light units/mg protein, day 26: 7.9 ± 3.4 relative light units/mg protein, untransfected: 0.01 relative light units/mg protein, $n = 3$/group) and **Figure 6**. Importantly, gene transfer occurred without the need for preconditioning.

Transduction with F/HN-SIV carrying the *CFTR* complementary DNA leads to expression of cAMP-dependent chloride channels

Iodide efflux is commonly used to demonstrate the presence of forskolin-activated chloride channels *in vitro*. In this assay, iodide

is used as a surrogate for chloride due to the shorter half-life of the radioactive material. We have constructed an F/HN-SIV carrying a GFP-*CFTR* fusion complementary DNA construct and transduced human embryonic kidney (HEK)293T cells to assess whether functional CFTR chloride channels are generated. Fusion of GFP to the N-terminus of CFTR has previously been shown not to effect CFTR function.[26,27] Figure 7 shows that cells transduced with F/HN-SIV-GFP-CFTR (multiplicity of infection 50) significantly ($P < 0.001$) increased cAMP-mediated efflux compared to cells treated with an F/HN-SIV-GFP control virus. We, therefore, conclude that transduction with F/HN-SIV-GFP-CFTR generates functional CFTR chloride channels *in vitro*.

DISCUSSION

Here, we show that (i) we can produce titers of a novel SIV vector pseudotyped with SeV envelope proteins appropriate for *in vivo* use, (ii) the vector can transduce the respiratory epithelium of the murine nose *in vivo* at levels that may be relevant for clinical benefit in CF, as previously suggested by *in vitro* mixing experiments,[28] (iii) this can be achieved in a single formulation, and without the need for preconditioning, (iv) expression can last for at least half the lifespan of a mouse, (v) the vector can produce levels of gene expression ~500-fold greater than the current optimal nonviral formulation after three repeated administrations, (vi) the vector is able to transduce a fully differentiated human airway epithelium, and (vii) can produce functional cAMP-dependent CFTR chloride channels *in vitro*.

We inserted cPPT and WPRE sequences into the SIV vector. Both elements have previously been reported to increase gene transduction efficiency possibly due to acceleration of the movement of the preintegration complex of the vector into the nucleus,[22,29,30] or other mechanisms.[23,31] Interestingly, a synergistic effect of these two elements has been observed for human immunodeficiency virus–based lentiviral vectors.[24] Our data are in keeping with these observations, with simultaneous insertion of cPPT and WPRE increasing productivity of both the VSV-G and SeV-F/HN-pseudotyped SIV vectors. Using these methods, we were able to reach titers of the SeV-F/HN-pseudotyped SIV vector of 5×10^7 TU/ml. Thus, this vector may be able to overcome one previously encountered important translational hurdle.

Lentiviral vectors pseudotyped with a variety of envelope proteins other than VSV-G have been described, including those from Ebola, Zaire,[15,16] influenza hemagglutinin from fowl plague virus,[18] and baculovirus GP64 envelopes.[17] Among these, arguably it is the transduction efficiency of the baculovirus GP64-pseudotyped vectors that is most impressive, when applied in a viscoelastic gel formulation (1% methylcellulose) as a vector solvent. However, the regulatory complexities of moving two new agents into the clinic simultaneously, underline the encouraging transduction efficiency and duration we report here without the need for additions to the formulation.

The likely target for CF gene therapy are the ciliated epithelial cells, and >70% of the cells transduced by the F/HN SIV vector were of this type. This is in keeping with transduction of these cells by the "parent" SeV vector, and overcomes a second hurdle in the translation of these vectors toward the clinic. The number of cells requiring transduction for clinical benefit is a vexed, and unresolved question. In part, this may depend on which of the many functions of CFTR requires correction. Thus, if the chloride channel function predominates, *in vitro* data suggest that as few as 5% of cells may be sufficient.[28] These values are in reach of the F/HN SIV vector described here.

Using a human cytomegalovirus promoter, we saw expression of GFP for >360 days in 16 out of 17 mice, although gene expression gradually decreased over time when quantified as number of cells expressing the GFP reporter gene or longitudinal assessment of bioluminescent imaging. The onset of transgene expression was typically delayed, with no GFP fluorescence detected at day 3, but clearly visible by day 10. This has also been reported for an Ebola virus Z protein–pseudotyped human immunodeficiency virus vector.[15] Possible explanations include the delayed movement of the preintegration complex to the nucleus, or the shutting off of promoter activity by concomitant inflammation consequent upon transduction,[32,33] with subsequent expression following the resolution of inflammation. Irrespective, transgene expression was still apparent up to 449 days after transduction the longest time point assessed. Because the lifespan of terminally differentiated airway epithelial cells has been estimated at around 90 days (ref. 6), both in mice and in man, we considered whether this vector may have transduced progenitor or stem cells within the airway epithelium. We would predict that in this case we would observe clonal expansion, with clustering of transgene positive cells, and this was seen following induced regeneration of epithelial cells after polidocanol treatment. Cells derived from progenitor or stem cells thought to reside near the basement membrane have previously been shown to move laterally during differentiation.[34] Thus, in the absence of epithelial damage, SIV vector–transduced progenitor or stem cell–derived cells should be observed in a scattered pattern without clustering (see schematic presentation in Figure 8a). In contrast, following epithelial stripping, a different pattern would be predicted to occur, with clusters of transduced cells becoming visible (see schematic presentation in Figure 8b). This hypothesis may explain, why we observed clusters of GFP-positive cells in damaged but not in undamaged epithelium. However, more extensive studies will be necessary to understand and more conclusively prove stem or progenitor cell transduction. Other explanations for the unexpectedly long duration of expression include (i) an alteration in the cell cycle of transduced respiratory epithelial cells, although to our knowledge this has not previously been reported, (ii) expression from -resident airway inflammatory or immune cells, for which we saw no evidence, or (iii) a >3 months half-life of respiratory epithelial cells, which has recently been suggested by Rawlins *et al.*[7]

The treatment of CF will require lifelong expression of the normal CFTR protein. Thus, despite these encouraging data showing long-lasting expression from a single administration, we assessed whether repeated application of this vector could sustain gene expression. We show that repeated mucosal administration of F/HN pseudotyped lentivirus, when given monthly over a 3-month period, is feasible and led to gene expression ~40% of that seen following a single administration. This dosing interval may be of subsequent clinical relevance. Sinn *et al.*[35] have recently shown that seven weekly administrations of a GP64-pseudotyped FIV, given in tandem with a ciliastatic agent, are able to produce repeatable expression. Further, each study was undertaken in a different inbred mouse strain. The significant differences between

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the studies, yet with similar outcome, provide a growing body of evidence that such vectors can be readministered.

Differentiated human airway epithelium is in general difficult to transduce. However, we have shown here that F/HN-SIV transduced fully differentiated human airway epithelium successfully, and that reporter gene expression could be detected for at least 26 days after transduction. Importantly, gene transfer occurred without the need for preconditioning with tight junction openers or cilia static agents that are often required with other viral vectors. This provides encouraging support for its use in human trial.

In addition to demonstrating that F/HN-SIV carrying the *CFTR* complementary DNA was able to generate cAMP-dependent chloride channels *in vitro*, we also attempted to correct nasal potential difference in CF knockout mice. However, we did not detect any changes in ion transport (data not shown). Importantly, the suitability of the CF mouse nasal epithelium as a model has been put into question by two recent publications showing that the nasal bioelectrics are dominated by the olfactory rather than the respiratory epithelium.[36,37] Our experience is in keeping with this observation. Transduction with SeV, which transduces respiratory and olfactory epithelium led to significant increases in chloride transport,[38] whereas lentivirus and nonviral gene transfer agents,[39] which predominantly transduce ciliated respiratory epithelial cells were unable to alter ion transport in the mouse nose. Until more appropriate animal models become widely available, analysis of *CFTR* function after gene transfer may, for certain gene transfer agents, be restricted to *in vitro* models.

Clearly, at least one remaining crucial hurdle is the risk–benefit ratio of these integrating vectors. The cases of leukemia in the severe combined immunodeficiency trial using a retroviral vector have been well documented, but lentiviral vectors are considered by many to be less susceptible to these problems. Further, the slowly dividing airway epithelium may represent a very different risk to the rapid turnover of bone marrow stem cells. Encouragingly, in our study of ~100 mice, over a 1-year period we saw no adverse events attributable to the vector. However, the encouraging increase in median survival of CF patients to the current ~36 years suggests that extensive toxicology studies will be needed before clinical trials can begin.

In conclusion, we suggest that the SeV-F/HN-pseudotyped SIV vector reported here may represent a further step toward translating such integrating viral vectors into clinical use. Several key hurdles have been potentially overcome, pushing these vectors into the arena as candidates for clinical trials.

MATERIALS AND METHODS

Cell culture. HEK293T and 293T/17 cells (CRL-11268; ATCC, Manassas, VA) were maintained in Dulbecco's minimal Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

Plasmid construction. pCAGGS-Fct4 and pCAGGS-SIVct+HN were constructed as previously described.[14] The cPPT and WPRE sequences[40] were inserted in the SIV-derived gene transfer plasmid.[21]

Production of SIV vector. Replication-defective self-inactivating SIV vector was constructed as previously described[20] with minor modifications. Briefly, the SeV-F/HN-pseudotyped SIV vector was produced by transfecting 293T/17 cells (15 cm diameter culture dishes) with four plasmids complexed to Lipofectamine/Plus reagents (Invitrogen) according to the manufacturer's recommendations [Plasmid-1: 10 μg SIV-derived transfer plasmid carrying a GFP, a luciferase (lux) reporter gene, or a GFP-*CFTR* fusion construct,[26] Plasmid-2: 3 μg packaging plasmid, Plasmid-3: 2 μg pCAGGS-Fct4, Plasmid 4: 2 μg pCAGGS-SIVct+HN]. The VSV-G pseudotyped SIV vector was produced using a similar protocol, but a pVSV-G plasmid (2 μg; Clontech, Mountain View, CA) was used instead of pCAGGS-Fct4 and pCAGGS-SIVct+HN. At 12 hours after transfection the culture medium was replaced with 30 ml serum-free Dulbecco's modified Eagle medium containing 5 mmol/l sodium butyrate. Sodium butyrate stimulates the vector production to inhibit histone deacetylase.[41,42] The culture supernatant containing the SIV vector was harvested 48 hours after transfection, filtered through a 0.45 μm filter membrane, and further concentrated by high-speed centrifugation (20,000 g for 4 hours at 4 °C, Avanti JA18 rotor; Beckman Coulter, Brea, CA). The vector pellets were suspended in PBS (Invitrogen) to 100- to 200-fold concentration and stored at −80 °C.

Vector titration. The particle titer was determined using real-time reverese transcriptase-PCR. Virus RNA was purified using a QIAamp viral RNA mini-kit (QIAGEN, Strasse, Germany), and reverse transcribed using Superscript II (Invitrogen). The QuantiTect probe PCR system (QIAGEN) and primers for amplifying 131 nucleotides (bp) spanning the WPRE sequence (forward primer: 5′-ggatacgctgctttaatgcc-3′, reverse primer: 5′-acgccacgttgcctgacaac-3′) were used according to the manufacturer's protocol in an ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA). SIV gene transfer plasmid DNA (3×10^4 to 2×10^6 molecules) was used as standard.

Transduction units (TU/ml) were determined by transducing 293T cells with serial dilutions of vector stock and quantification of transduced cells by GFP fluorescence (for F/HN-SIV-GFP and VSV-G-SIV-GFP) or staining with anti-luciferase antibody (for F/HN-SIV-lux). To work with a consistent virus concentration throughout the study, virus stocks were all adjusted to a final volume of 4×10^9 TU/ml. A titer of 4×10^9 TU/ml was used for all animal studies.

In vivo administration to the mouse nose. All animal studies had been approved by the DNAVEC Animal Care Committee and the Imperial College Animal Ethics Committee and were carried out according to Home Office regulations. C57BL/6N mice (female, 6–8 weeks) were used. Mice were anesthetized, placed horizontally on their backs onto a heated board, and a thin catheter (<0.5 mm outer diameter) was inserted ~2.5 mm from the tip of nose into the left nostril. Using a syringe pump (Cole-Parmer, Vernon Hills, IL), vector (100 μl) was then slowly perfused onto the nasal epithelium (1.3 μl/min) for 75 minutes. Despite perfusion of virus into the left nostril, we routinely observe transfection in both left and right nostrils, which is due to dispersion of the solutions throughout the entire nasal cavity. PBS and VSV-G-SIV transduced mice preconditioned with 1% lysophosphatidylcholine as described by Limberis *et al.* were used as controls.[11] At indicated time points (3–360 days after transduction), mice were culled to visualize GFP expression.

In the 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 30 days after F/HN-SIV-lux administration. For comparison, mice were transfected with the cationic lipid GL67A complexed to a luciferase reporter gene as previously described[43] and luciferase expression was measured 2 days after transfection.

Induced regeneration of nasal epithelial cells by polidocanol treatment. Nasal epithelial cells were stripped by polidocanol treatment according to

the method described by Borthwick *et al.*[6] with some modification. In brief, mice were anesthetized and 10 μl polidocanol (2%) were administered to the nose as a bolus by "nasal sniffing". To confirm the stripping and regeneration of nasal epithelial cells, nasal tissue was perfused with 10 μl of 2% (vol/vol in PBS) polidocanol (nonaethylene glycol mono-dodecyl ether; SIGMA, St Louis, MO) and histological analysis undertaken 24 hours and 7 days after treatment ($n = 3$/group). To analyze transduction of possible progenitor or stem cells, we first administered F/HN-SIV-GFP (4×10^8 TU/mouse) vector to mouse nasal epithelium. Seven days after transduction, nasal tissue was perfused with 10 μl of 2% (vol/vol in PBS) polidocanol, and this treatment was repeated again 3 weeks later. Histological sections were analyzed 58 days after vector administration (30 days after the last polidocanol treatment).

Bioluminescent imaging. Mice were injected intraperitoneally with 150 mg/kg of D-luciferin (Xenogen, Alameda, CA) 10 minutes before imaging and were anesthetized with isoflurane. Bioluminescence (photons/s/cm²/sr) from living mice was measured using an IVIS50 system (Xenogen) at a binning of 4 for 10 minutes, using the software programme Living Image (Xenogen). For anatomical localization a pseudocolor image representing light intensity (blue: least intense, red: most intense) was generated using Living Image software and superimposed over the grayscale reference image. To quantify bioluminescence in the nose, photon emission in a defined area (red box) was measured by marking a standardized area for quantification. The size of the red box was kept constant and was placed over the heads of the animals as indicated in the figure. Importantly, the areas were marked using the grayscale reference image to avoid bias.

Tissue preparation for histological assessment of GFP expression. Mice were culled and the skin was removed. The head was cut at eye level and skin, jaw, tongue, and the soft tip of the nose were carefully removed. For *in situ* imaging of GFP expression in the nasal cavity, GFP fluorescence was detected using fluorescence stereoscopic microscopy (Leica, Ernst Leitz Optische Werke, Germany). Subsequently, the tissue was fixed in 4% paraformaldehyde (pH 7.4) overnight at room temperature and was then submerged in 20% EDTA (pH 7.5 for 5 days) for decalcification. The EDTA solution was changed at least every second day. After decalcification, the tissue was incubated in 15% sucrose overnight at room temperature and was then embedded in Tissue Mount (Chiba Medical, Soka, Japan). Ten micrometer sections were cut at six different positions in each mouse head (~0–6 mm from the tip of nasal bone). GFP expression was observed using a fluorescent microscope (Leica). Quantification and identification of cell types were carried out on six levels per mouse using a ×40 or ×63 objective. Prolonged image exposure was necessary to capture the structure of the nasal epithelium using fluorescent microscopy. This led to pixel saturation of GFP-positive cells and caused GFP-positive cells to appear almost white rather than the common green appearance that we, and others, observe under higher magnification.

Transduction of ALI cultures. Fully differentiated airway epithelial cells grown as ALI cultures were purchased from Epithelix (Geneva, Switzerland). ALIs were transfected with F/HN-SIV-lux at a multiplicity of infection ranging from ~25 to ~300. The virus was dissolved in 50 μl PBS and applied to the apical surface. After 6 hours, the virus was removed and ALIs were incubated for 10–26 days. The basolateral medium was changed every 48 hours during this incubation period. At specified time points, the ALIs were lysed in 100 μl reporter lysis buffer and luciferase expression was quantified using the Luciferase Assay System (Promega, Southampton, UK) according to the manufacturer's instructions. The total protein content of the cultures was quantified using the BioRad protein assay kit (BioRad, Hemel Hempstead, UK). Each sample was assayed in duplicate. Luciferase expression was then presented as relative light units/mg total protein. For bioluminescence imaging 100 μg luciferin in PBS were added to the apical membrane.

Iodide efflux assay. HEK293T cells were transfected with F/HN-SIV-GFP-CFTR or an F/HN-SIV-GFP control virus at a multiplicity of infection of 500 and cultured for 2 days. CFTR chloride channel activity was assayed by measuring the rate of ^{125}iodide efflux as previously described.[44] The ^{125}iodide efflux rates were normalized to the time of forskolin/IBMX addition (time 0). Curves were constructed by plotting rates of ^{125}iodide efflux against ime. To reflect the cumulative levels of ^{125}iodide efflux following agonist-stimulation, all comparisons are based on areas under the time-^{125}iodide efflux curves. The area under the curve was calculated by the trapezium rule. Experiments were carried out in duplicate ($n = 6$ wells/group/experiment).

Statistical analysis. Normal distribution was assessed for all data and parametric or nonparametric statistical analysis was performed as appropriate. Data in Figure 3f were analyzed using the Mann–Whitney U-test to compare bioluminescence at month 1 and 8, as appropriate for nonparametric data. In 2 out 10 mice, bioluminescence had returned to baseline levels before month 8 and these mice did not undergo additional bioluminescence *in vivo* imaging. For the final quantification of gene expression at 8 months the mean bioluminescence of the PBS control cohort was used for these two mice. In addition, data in Figure 3f were analyzed using a Friedman repeat measure test followed by Dunn's multiple comparison *post hoc* test, as appropriate for nonparametric data.

Data in Figure 5 were analyzed by Kruskal–Wallis followed by Dunn's Multiple Comparison test, as appropriate for nonparametric data. Data in Figure 6 were analyzed by ANOVA followed by Bonferroni's Multiple Comparison *post hoc* test, as appropriate for normal distributed data. The null hypothesis was rejected at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Figure S1. SeV F and HN envelope proteins are incorporated into SIV vector particles and form infectious SIV pseudotyped vectors.
Figure S2. GFP-positive cluster in F/HN-SIV-GFP transduced and polidocanol-treated mouse nasal epithelium.
Materials and Methods.

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ORIGINAL ARTICLE *Clinical haemophilia*

Continuous infusion during total joint arthroplasty in Japanese haemophilia A patients: comparison study among two recombinants and one plasma-derived factor VIII

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Summary. As for the available factor VIII (FVIII) concentrates in Japan, there are two recombinant FVIII concentrates (Kogenate-FS and Advate) and one highly purified plasma-derived FVIII concentrate (Cross-Eight M). To evaluate the inter-product variability, the differences in the continuous infusion rates and total consumption of the above three concentrates were compared when continuous infusion was used as the administration mode to control bleeding during 28 total joint arthroplasties (TJAs) for 17 patients. There were no significant differences among the FVIII plasma levels during surgery, except day 0. Advate needed to be given at a significantly higher infusion rate (4.2–2.1 IU kg^{-1} h^{-1}) than the other two concentrates (Kogenate-FS: 1.0–2.9 IU kg^{-1} h^{-1}, $P < 0.01$ and $P < 0.05$; Cross-Eight M: 3.2–1.8 IU kg^{-1} h^{-1}, $P < 0.01$); however, their infusion rates were within the rates which were previously reported. The total consumption of Advate (652.1 IU kg^{-1}) was also significantly greater than either of the other concentrates (Kogenate-FS: 395.1 IU kg^{-1}, $P < 0.01$; Cross-Eight M: 519.1 IU kg^{-1}, $P < 0.05$). The results of this study showed that the continuous infusion of three FVIII concentrates is effective and safe during TJA, and also showed the differences in the continuous infusion rates and total consumption among concentrates when continuous infusion was used to control bleeding during surgery. These two results suggested that the continuous infusion of FVIII concentrate is a good administration mode, but there is still room for further investigation to use it as a more cost-effective and safer administration mode.

Keywords: continuous infusion, haemophilia A, inter-product variability, total joint surgery

Introduction

There are many *in vitro* and *in vivo* studies on the complications of continuous infusion of factor VIII (FVIII) such as, stability after reconstitution, biological sterility, thrombophlebitis and inhibitor formation [1–8], and there is still room for further investigation [9]. However, continuous infusion is known worldwide as a cost-effective and safe administration mode, because this mode can be used to avoid unnecessary high-peak factor levels and unexpected low-trough levels.

Also, many clinical success results were reported using continuous infusion [1–5]. In those reports, the continuous infusion speed was from 2.0 to 6.0 IU kg^{-1} h^{-1} when the desired factor level was aimed at 1.0 IU mL^{-1}. However, no consideration was given to the different states in which bleeding occurs, such as trauma, major surgery and minor surgery, when attempting to achieve haemostasis, nor were the differences in individual concentrate efficacy considered during the administration of continuous infusion. In other words, there are no reports focused on inter-product variability, or inter-individual variability during continuous infusion.

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As for the available concentrates in Japan, there are two recombinant FVIII concentrates: Kogenate-FS® (Bayer Healthcare, Berkeley, CA, USA) and Advate® (Baxter Healthcare Corporation, Westlake Village, CA, USA), and one highly purified plasma-derived FVIII: Cross-Eight M® (Japanese Red Cross Society, Chitose, Hokkaido, Japan). In this study, to evaluate the inter-product variability, the differences in the above three concentrates were compared when continuous infusion was used as the administration mode to control bleeding during total joint arthroplasty (TJA).

Patients and methods

In total 66 orthopaedic surgeries for 43 haemophilic patients were performed between April 2006 and August 2009 at one centre: 46 surgeries for 29 haemophilia A patients, nine surgeries for seven haemophilia B patients and 11 surgeries for seven haemophilia patients with inhibitors. Among the surgeries for haemophilia A, 28 TJAs for 17 severe haemophilia A Japanese patients without inhibitors were performed using three kinds of concentrates to control bleeding during surgery. Those concentrates are the available concentrates in Japan; Kogenate-FS® (Bayer Healthcare), Advate® (Baxter Healthcare Corporation) and Cross-Eight M® (Japanese Red Cross Society). All concentrates were labelled at 1000 IU per vial. The type of concentrate used during surgery was chosen based on the type previously used by the patient, and never exchanged with another concentrate.

A simple pharmacokinetics study was done for all patients before the first operation, and the FVIII *in vivo* recovery (IVR) and half-life were calculated. The FVIII activity (FVIII:C) was measured in our hospital by a one-stage activated thromboplastin time assay using the CA-1500 analyzer (Sysmex, Hyogo, Japan) before and at 30 min, 6 and 24 h after a 1000 IU concentrate was injected. The FVIII IVR (IU dL^{-1}) per (IU kg^{-1}) was defined as the ratio of FVIII:C increment to the units per kilogram infused of FVIII, and the half-life (h) is the length of time it takes for a 50% drop in plasma drug concentration as one compartment model in this study.

A bolus infusion of FVIII concentrate at a dose calculated to raise the plasma level to 80–100% was administered 30–60 min before the start of the surgery. The plasma FVIII level was confirmed at 10–20 min after bolus injection. At the same time, continuous infusion was initiated at a rate calculated to maintain a steady concentration of 80–100%. The bolus infusion dose of FVIII concentrate and initial concentrate infusion rate were determined according to the results of the simple pharmacokinetics study. Continuous infusion was maintained until day 6, and the daily continuous infusion dosage was adjusted throughout surgical periods to maintain a FVIII:C level of 80% for the first 2 days, 60–80% until day 4 and 40–60% until day 6 at surgery. As a rule, the plasma FVIII level was checked at 30 min, 3 h after surgery, day 1 or 2, day 3 or 4, day 5 or 6 and day 7 at surgery. Based on the FVIII levels at three points at day 0, the actual continuous infusion rate was determined to maintain a steady concentration of 80–100%. Also, the daily continuous infusion rate was determined based on the individual continuous infusion rate at day 0. The factor levels after day 0 were measured to confirm that the individual continuous infusion rate maintained the target FVIII level of the haemostatic protocol, and minor adjustments were done periodically.

Reconstituted FVIII concentrate in a 50 mL polypropylene syringe pump (Terumo, Tokyo, Japan) and a polyvinyl chloride extension tube that has a volume of 0.5 m: (Terumo) were connected parallel to a lactic ringer fluid drip system (drip speed: 500 mL per 24 h). It was infused continuously and exchanged within 12 h to maintain the stability and sterility of reconstituted FVIII at room temperature. The three concentrates were used without heparin, and none were diluted except for Kogenate-FS. Kogenate-FS was diluted (×4) with distilled water to avoid thrombophlebitis [10]. After all data were confirmed to be normally distributed by the Shapiro–Wilk test, they were analysed by the Bonferroni test (multiple comparisons) as parametrical data. SPSS (version 15.0, 2006, Troy, NY, USA) was used for all statistical analysis.

Results

Patient parameters

Patient parameters are shown in Table 1. All patients were adult severe haemophilia A Japanese patients, and there were no significant differences in other parameters among the three concentrates, except platelet counts. None of the patients in this study had inhibitors for FVIII before and after surgery.

Pharmacokinetic analysis and product factor activity

The average FVIII IVR was 1.86 ± 0.4 (IU dL^{-1}) per (IU kg^{-1}), and there were no significant differences among the three concentrates (Table 2). Also, the

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Table 1. Patient and surgical parameters.

	Unit	*n*	Kogenate-FS	*n*	Advate	*n*	Cross-Eight M	*n*	Total	Statistical analysis
Age	Year	7	39.7 ± 12.9	9	44.3 ± 7.5	12	42.2 ± 8.8	28	42.3 ± 9.4	*ns*
Body weight	kg	7	54 ± 4.0	9	58.7 ± 11.1	12	61.7 ± 6.1	28	58.8 ± 8.0	*ns*
Platelet	10^4 μL	7	23.7 ± 6.1	9	17.9 ± 4.2	12	15.4 ± 7.9	28	18.3 ± 7.1	*
Blood loss	mL	7	1120.4 ± 470.2	9	868.4 ± 361.1	12	933.2 ± 326.7	28	959.2 ± 375.5	*ns*
Transfusion volume	mL	7	620.0 ± 293.3	9	482.2 ± 310.9	12	530 ± 344.0	28	537.1 ± 314.3	*ns*
TKA (re-TKA)	Case		4		8		9 (1)		21 (1)	
THA (re-THA)	Case		3				2 (1)		5 (1)	
TEA	Case				1				1	
BHA	Case						1		1	
Hepatitis C virus positive	Case	7	5	9	9	12	12	28	26	
HIV positive	Case	7	5	9	6	12	3	28	14	
HAART	Case	5	2	6	2	3	3	14	7	

ns, no significant differences among the three concentrates; TKA, total knee arthroplasty; THA, total hip arthroplasty; TEA, total elbow arthroplasty; BHA, bipolar hip arthroplasty; Re-TKA, revision TKA; Re-THA, revision THA; HAART, highly active retroviral therapy.
*$P < 0.05$ comparison between Kogenate-FS and Cross-Eight M.

Table 2. Pharmacokinetic analysis and product factor activity.

	Unit	Kogenate-FS	Advate	Cross-Eight M	Total	Statistic
Sample	Counts	11	6	11	28	
IVR	(IU dL^{-1}) per (IU kg^{-1})	1.91 ± 0.4	1.86 ± 0.5	1.83 ± 0.2	1.86 ± 0.4	*ns*
Half-life	h	13.7 ± 3.2	11.7 ± 4.0	13.2 ± 3.5	13.1 ± 3.5	*ns*
Batches	Counts	15	14	21		
Activity	IU per vial	1147 ± 47.4	1041 ± 34.7	1135.2 ± 39.1	1112 ± 60.3	*,†

ns, no significant differences among the three concentrates; IVR, *in vivo* recovery.
*$P < 0.01$ comparison between Kogenate-FS and Advate.
†$P < 0.01$ comparison between Advate and Cross-Eight M.

average half-life was 13.1 ± 3.5 (h), and there were no significant differences among the three concentrates. Several different batches of concentrates were used for surgery; 15 batches of Kogenate-FS® (Bayer Healthcare), 14 batches of Advate® (Baxter Healthcare Corporation) and 21 batches of Cross-Eight M® (Japanese Red Cross Society). The batches differed according to the time the operation was performed. Those product FVIII activity data were obtained from the product companies. The average activity of Advate was significantly lower than the average activity of Kogenate-FS and Cross-Eight M. Compared with the labelled activity on the vial, the average activity of Advate was as labelled, but the average activity of Kogenate-FS and Cross-Eight M was approximately 1.1 times as labelled.

Factor VIII:C (%) and concentrate doses

The actual FVIII plasma level at peri-operative periods was close to the target FVIII level according to the above haemostatic plan. The FVIII plasma levels of the Kogenate group were significantly higher than that of the others at just the first two measurement points, and there were no significant differences among the three concentrates later (Fig. 1).

To reduce the differences in product variability among the three concentrates, the original bolus infusion dose and continuous infusion rate of Kogenate-FS and Cross-Eight M were adjusted according to the differences in product activity. Even after adjustments were made to the bolus infusion dose and continuous infusion rate, there were significant differences among the three concentrates. There were no significant differences among the bolus infusion doses of the three FVIII concentrates (Kogenate-FS: 61.2 ± 11.8; Advate: 65.1 ± 10.1; Cross-Eight M: 62.5 ± 7.6) calculated to raise the plasma level to 80–100%. However, the continuous infusion rate of Advate (2.1–4.2 IU kg^{-1} h^{-1}: average range during postoperative 7 days) was significantly higher than that of Kogenate-FS (1.0–2.9 IU kg^{-1} h^{-1}, $P < 0.01$ and $P < 0.05$) and Cross-Eight M (1.8–3.2 IU kg^{-1} h^{-1}, $P < 0.01$) to maintain the target

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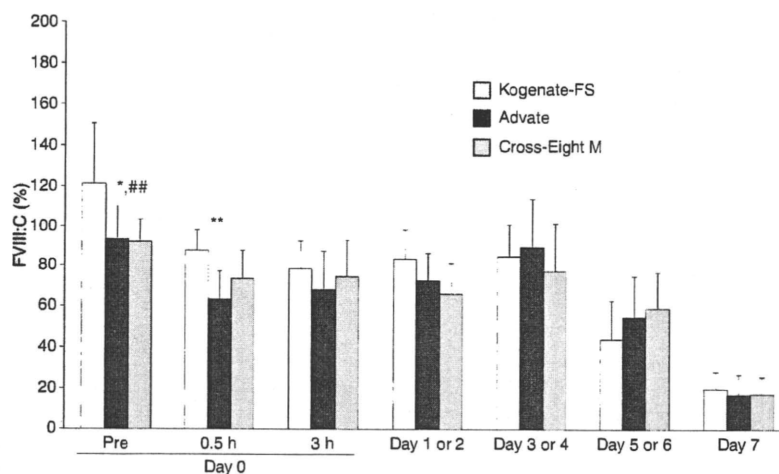

Fig. 1. Factor VIII (FVIII) levels during peri-operative periods. FVIII levels of the Kogenate group at preoperation and 0.5 h after operation were siginificantly higher than that of other concentrate cases (* $P < 0.05$; ** $P < 0.01$ compared with Advate; ## $P < 0.01$ compared with Cross-Eight M).

plasma level (Fig. 2). Also, the total consumption of Advate (652.1 ± 183.0 IU kg^{-1}: average) was significantly greater than either of the other concentrates (Kogenate-FS: 395.1 ± 65.0 IU kg^{-1}, $P < 0.01$; Cross-Eight M: 519.1 ± 68.0 IU kg^{-1}, $P < 0.05$).

Surgical parameters

Various types of TJAs were performed for patients, but there were no significant differences in blood loss from the period during surgery until day 2 among the three concentrates (Table 1). Between day 0 and day 2, unexpected bleedings and drops in FVIII levels occurred during eight surgeries. As a result, one injection of concentrate was required to control bleeding and raise the plasma level. For Kogenate-FS, one total knee arthroplasty (TKA) required one extra bolus injection. One TKA, one total hip arthroplasty (THA), one revision TKA, and one revision THA for Cross-Eight M, and three TKAs for Advate also required one extra bolus injection.

For all patients, 400 g of whole blood was pooled several days before surgery, and then returned to the patient at day 0 or day 1 as an autologous blood transfusion. Drainage blood from the wound was also returned to the patient by using the ConstaVac CBC II Blood Conservation System (Stryker, Mahwah, NJ, USA). Homologous blood was transfused for two cases; one THA using Kogenate-FS, and one TKA using Cross-Eight M. There were no differences in the total transfusion volumes among the three concentrates.

Other optional treatments

A tourniquet was not routinely used for TKA, instead, strict protocols were followed to coagulate bleeding during surgery to avoid complications, such

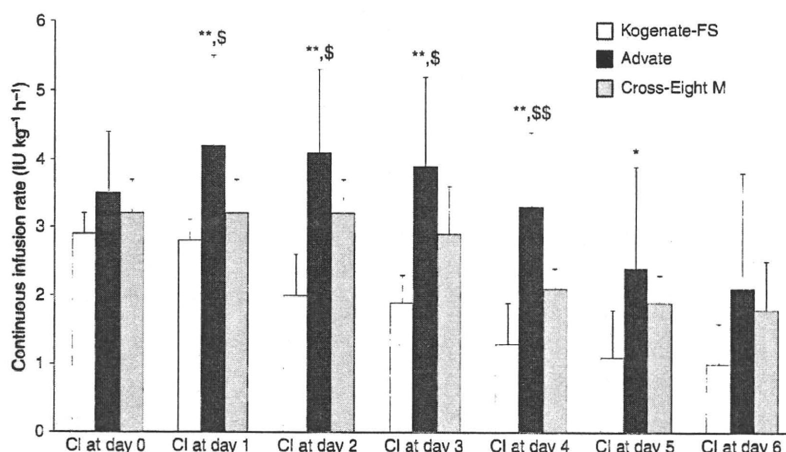

Fig. 2. Continuous infusion rate. The continuous infusion rate of Advate is significantly greater than that of other concentrates (* $P < 0.05$; ** $P < 0.01$ compared with Kogenate-FS; $ $P < 0.05$; $$ $P < 0.01$ compared with Cross-Eight M).

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as deep vein thrombosis, after surgery. Tranexamic acids were not used for any of the patients.

Other adverse effects

Based on appearance, the reconstituted concentrate in the infusion system was clear, and there were no signs of bacterial contamination. Also, none of the patients showed any signs of inhibitors, thrombosis or thrombophlebitis.

Discussion

Continuous infusion of FVIII was described already in 1954 [11] and proposed in 1970 as an effective alternative to the traditional bolus infusion to provide a safe, steady-state haemostatic factor level [12]. Many pharmacokinetics and biological studies, *in vivo* and *in vitro*, confirmed the efficacy, stability and microbiological safety of reconstituted FVIII at room temperature for at least 24 h. Neidhardt *et al.* [6] reported the stability of B-domain deleted recombinant FVIII within 72 h after reconstitution *in vitro*. Parti *et al.* [7] also reported the stability of plasma/albumin-free recombinant human FVIII at room temperature within 24 h after reconstitution *in vitro*. Stachnik reviewed clinical reports about continuous infusion of factor products for haemophilia and concluded that factor products were stable and sterile for periods longer than several hours [8]. Martinowitz *et al.* [4] reported that continuous infusion of sucrose-formulated recombinant FVIII is stable, effective and safe during major surgery within 24 h. Lee *et al.* [3] described that continuous infusion was a cost-effective delivery system with the portable minipumps, as approximately 30% of the total consumption of the concentrate can be saved, when compared with intermittent bolus infusion. Also, many comparison clinical studies between bolus infusion and continuous infusion [1,2,5] made clear some additional advantages of continuous infusion; avoidance of unnecessary high-factor peak levels and unexpected low-trough levels, and reduction of factor consumption at haemostasis. In this study, continuous infusion was also a safe and effective administration mode, because there were no adverse effects, such as biological infection, or an unexpected drop in FVIII activity.

However, it was difficult to judge the continuous infusion rate and clearance. The initial infusion rate in previous studies [2,5,13–15] was from 1.8 to 6.0 IU kg^{-1} h^{-1}, and the average clearance of FVIII in previous studies [1,4,16] was from 2.65 to 3.89 mL kg^{-1} h^{-1}. In the above results, there are only three reports that described the clearance and infusion rate for a single product during some kinds of surgeries. Stieltjes *et al.* [15] described that the initial infusion rate of ReFacto® (Wyeth Pharmaceuticals Inc., Philadelphia, PA, USA) was from 1.8 to 6.0 IU kg^{-1} h^{-1} during 20 surgical procedures. Negrier *et al.* [5] also described that the initial continuous infusion rate of Advate was from 3.2 to 3.7 IU kg^{-1} h^{-1} during 18 surgical procedures for major and minor orthopaedic surgeries, and non-orthopaedic surgeries. Martinowitz *et al.* [4] described that the clearance of Kogenate-FS was 3.02 mL kg^{-1} h^{-1} (1.6–4.6 mL kg^{-1} h^{-1}) according to a pharmacokinetics study. In this study, the average for the initial continuous infusion rate of Kogenate-FS, Advate, and Cross-Eight M was 2.9 ± 0.3, 3.5 ± 0.9, and 3.2 ± 0.5 IU kg^{-1} h^{-1}, respectively, during TJA. This result showed the efficacy and safety of continuous infusion of these three concentrates, because their initial infusion rates were in the range of the above previous results.

As for the causes of influence on continuous infusion rates, many factors are nominated such as, the adsorption in the infusion system, the dilution of concentrate, and inter-product, inter-individual, and inter-surgical procedure variations.

Henze *et al.* described that the activity of Kogenate-FS is reduced in polyvinyl bags within the first few hours, as a result of the adsorption of recombinant FVIII protein to the inner surface of the tubing and bags. However, the results suggested that the minimal binding of protein to the infusion tubing at the start of continuous infusion would probably not alter the plasma FVIII concentration in a clinical situation [17]. McLeod *et al.* [18] also pointed out that the amount of FVIII activity significantly decreased during storage in the polyvinyl chloride bags for Kogenate-FS, diluted from 146–2 IU mL^{-1}. In this study, Kogenate-FS maintained good factor activity even if it was diluted (×4) with distilled water and infused continuously by using a polyvinyl chloride delivery system. Fernandez *et al.* [19] researched the stability of Advate during simulated continuous infusion based on three delivery systems, factor potency (high or mid potency) and dilution, and concluded that Advate is stable under conditions typically encountered during continuous infusion and suggested that Advate should be safe and effective when used for FVIII replacement by continuous infusion. The volume (0.5 mL) of the polyvinyl chloride extension tube in this study seemed to be too small to influence the adsorption of a concentrate. However, in this study, the adsorption in this infusion system might have

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influenced the continuous infusion rate, because the IVR and half-life of Advate were the same as those of the other concentrates according to the simple pharmacokinetic study, but Advate required a higher infusion speed when infused continuously.

As for the inter-product variability, Hermans *et al.* [16] described that the clearance of recombinant FVIII (2.65 ± 0.8 mL kg^{-1} h^{-1}) was significantly lower than that of plasma derived FVIII (3.97 ± 1.38 mL kg^{-1} h^{-1}). On the other hand, Bidlingmaier *et al.* [2] described that the product type, such as plasma derived or recombinant factor concentrates, did not influence the infusion rate, and the median infusion rate was 4.4 IU kg^{-1} h^{-1} (range: 2.8–9.5 IU kg^{-1} h^{-1}). This study showed that the continuous infusion rate of Advate is significantly higher than that of Kogenate-FS and Cross-Eight M, and the total consumption of Advate is significantly larger than that of Kogenate-FS and Cross-Eight M. These results suggested that the clearance of Advate was more easily influenced by the surgical condition such as a massive vascular injury or exsanguinations, or the administration method such as continuous infusion, compared with the other concentrates.

All the previous studies discussed the continuous infusion rates without consideration given to the variability of inter-surgical procedures; however, in relation to continuous infusion, it is an important factor for discussion, as well as, inter-individual variability. Batorova and Martinowitz described that the possible inter-individual variations of clearance, according to age, bodyweight, laboratory assay employed and type of concentrate used, should be considered. And to prevent an unexpected drop in the factor level in a particular patient, because of higher than expected clearance, it is advised to check the factor level a few hours later and to increase the rate if necessary [16].

In this study, the variability of inter-individuality, inter-product activity and inter-surgical procedure was made as small as possible; however, there were significant differences in the consumption of concentrates. Schulman described that there is still room for further investigation concerning the complications of continuous infusion of FVIII concentrates, and that future studies should address the question of minimum steady-state levels required for haemostasis [9]. Further studies are needed to conclude what factors influence the infusion speed of FVIII concentrates when infused continuously.

This study showed that the continuous infusion of two recombinant factors and highly purified plasma-derived FVIII are effective and safe during TJA, because the continuous infusion rates of those three concentrates are in the usual infusion rate range. Importantly, Advate needed to be given at a significantly higher infusion rate than the other two concentrates. The total consumption of Advate was also significantly greater than either of the other concentrates. These two results suggested that the continuous infusion of FVIII concentrate is a good administration mode, but there is still room for further investigation to use it as a more cost-effective and safer administration mode.

Disclosures

The author stated that he had no interests which might be perceived as posing a conflict or bias.

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ORIGINAL ARTICLE *Paediatrics*

Major orthopaedic surgeries for haemophilia with inhibitors using rFVIIa

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Summary. Between 2000 and 2008, 11 major orthopaedic surgeries for 7 congenital haemophilia patients with inhibitors were performed by the first author as the primary doctor using recombinant activated factor VII (rFVIIa). Orthopaedic surgical treatments were performed for six surgeries for four high-responder haemophilia A patients, three surgeries for two high-responder haemophilia B patients and two surgeries for one low-responder haemophilia B patient. This low-responder patient is allergic to factor IX products, so he usually uses rFVIIa as a haemostatic agent. All of the surgeries were major, such as joint arthroplasty, arthroscopic synovectomy, and a combination of both, and excellent surgical results were achieved. Seven cases were controlled by bolus infusion of rFVIIa, and the other four cases were controlled by combined bolus and continuous infusion of rFVIIa. An anti-fibrolytic agent was used for all cases. There were no thrombogenic adverse effects, only two bleeding episodes. As for haemostatic control, nine surgeries were excellent, one was good and one was fair. This report is the largest clinical report on major orthopaedic surgeries at a single institute. We have concluded that the combination of bolus and continuous infusion of rFVIIa is safe and effective, and more convenient to administer than simple bolus infusion therapy to achieve haemostasis at peri-operative periods. In addition, our data also concurs with the data of several previous reports which showed that orthopaedic surgery for haemophilia patients with inhibitors by means of rFVIIa is safe and effective.

Keywords: inhibitor, orthopaedic surgery, rFVIIa

Introduction

For haemophilia patients without inhibitors, orthopaedic surgery is becoming popular. However, for haemophilia patients with antibodies for the deficient factor VIII or IX (inhibitor), this surgery is an elective treatment [1,2], because there is still no guideline, based on surgical case studies, which specifies a proper monitoring marker to monitor the coagulation factor plasma levels and how to effectively use concentrates to control bleeding during surgery. However, many haemophilia patients with inhibitors also complain about joint dysfunction and seek out orthopaedic surgical treatments. Surgical results were reported for some surgeries that were performed using bypassing agents, such as activated prothrombin complex concentrate (aPCC) and activated recombinant factor VII (rFVIIa). Most of these results were of minor surgeries such as radioactive synovectomy or tooth extraction. In some multicentre studies [1,3–6], the results of more than 10 major orthopaedic surgeries were reported, but there is no large report from a single treatment centre. This report includes the results of 11 major surgeries that were performed for 7 haemophilia patients by one orthopaedic surgeon as the primary doctor who is

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sufficiently knowledgeable in matters related to haemostasis and an experienced and active surgeon at two hospitals.

Patients

Between 2000 and 2008, the first author as the primary doctor performed 16 orthopaedic surgeries at 2 hospitals for 10 haemophilia patients with inhibitors. During 11 orthopaedic surgeries and postoperative periods for 7 patients, rFVIIa was used to control bleeding. Seven surgeries were performed at the first hospital between 2000 and 2006, and four surgeries were performed at the second hospital between 2007 and 2008. All surgeries were performed by one primary doctor, knowledgeable in haemostasis and an active surgeon, thus, making this the largest clinical report based on major orthopaedic surgeries at a single institute. For Case 1, there was a pseudotumour (20 × 10 cm) in the left femoral region with two skin ulcers. For Case 2, the abduction of both shoulders was less than 80° and flexion was less than 60° owing to osteophytic impingement. Total knee arthroplasty and bipolar hip arthroplasty (Cases 3 and 7) were indicated for end-stage arthropathy with severe pain and recurrent bleeding. Arthroscopic synovectomy (Cases 4, 5 and 6) was indicated for early or progressive arthropathy with recurrent bleeding.

Results

Six orthopaedic surgical treatments were performed for four high-responder haemophilia A patients, three surgeries for two high-responder haemophilia B patients and two surgeries for one low-responder haemophilia B patient. This low-responder patient is allergic to FIX products, so he usually uses rFVIIa as a haemostatic agent (Table 1). The average age at operation was 26 years (11–52 years old) and the average body weight was 56 kg (43–80 kg). The preoperative inhibitor titres were 1–54.3 Bethesda Units (BU) mL^{-1} and the plasma-deficient factor levels in all cases were less than 1%, except one (1.7%). All of the surgeries were major, such as joint arthroplasties, arthroscopic synovectomy and a combination of both (Table 2). Four arthroscopic knee synovectomies and two total knee arthroplasties were performed using a tourniquet. There was very little bleeding during the surgeries except for the one that was combined with septoplasty. The blood loss volumes for five orthopaedic surgeries performed for haemophilia patients with inhibitors, without the use of a tourniquet, were comparatively the same with the blood loss volumes for orthopaedic surgeries for haemophilia patients without inhibitors. The average operation period was 125.3 min (69–193 min).

The average of one bolus infusion dose per body weight was 113.6 mcg kg^{-1} (range 95.2–39.5 mcg kg^{-1}) (Table 3). For the first case (Case 1), which was our first experience, we administered the first bolus infusion before general anaesthesia, and then the second bolus infusion after bleeding occurred following the first incision. For cases that followed, we administered the first bolus infusion before general anaesthesia, and then the second bolus infusion just before the first incision. The average total period for peri-operative bleeding control using rFVIIa was 11.5 days (range 6–23 days). This period, compared with the first two cases (Cases 1 and 2-1), was longer than that of the other cases and the total dose per body weight was also gradually decreased. Bleeding complications during seven surgeries were controlled by bolus infusions of rFVIIa (bolus group) and the other four surgeries were controlled by combined bolus and continuous infusions of rFVIIa (combined group). The total period for peri-operative bleeding control of the bolus group

Table 1. Patient demographics and clinical characteristics: 11 major orthopaedic surgeries were performed for 7 congenital haemophilic patients with inhibitors.

Case	Age (years)	Body weight (kg)	Deficient factor	Deficient factor level (%)	Inhibitor titre (BU mL^{-1})	Anamnestic response
1	52	58	VIII	1.70	17.5	Yes
2-1	29	51	VIII	<1	2.3	Yes
3-1	38	60	VIII	<1	6.5	Yes
4-1	11	43	IX	<1	28.8	Yes
5	27	48	VIII	<1	1	Yes
3-2	39	60	VIII	<1	5.8	
6	13	45	IX	<1	54.3	Yes
4-2	13	47	IX	<1	29	
2-2	33	80	VIII	<1	13	
7-1	16	63	IX	<1	1	No
7-2	17	60	IX	<1	1	No

BU, Bethesda Unit.

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Case	Operation	Operation time (min)	Blood loss (mL)	Tourniquet
1	Removal of pseudotumour	193	1608	No
2-1	Removal of osteophytes and manipulation of right shoulder	107	57	No
3-1	Right total knee arthroplasty	146	120	Yes
4-1	Arthroscopic synovectomy for right knee	119	Little	Yes
5	Arthroscopic synovectomy for right knee and removal of osteophytes of left ankle	163	Little	Yes
3-2	Left total knee arthroplasty and septoplasty	160	610	Yes
6	Arthroscopic synovectomy for right knee	116	Little	Yes
4-2	Arthroscopic synovectomy for left knee	95	Little	Yes
2-2	Removal of osteophytes of left shoulder	106	53	No
7-1	Left bipolar hip arthroplasty	104	1056	No
7-2	Right bipolar hip arthroplasty	69	519	No

Table 2. Surgical data: seven surgeries from Cases 1 to 6 were performed at the former hospital and the others from Cases 4-2 to 7-2 were performed at the present hospital.

(average 12.3 days) was longer than that of the combined group (6.5 days), and the total dose per body weight of the bolus group (7.66 g kg^{-1}) was also more than that of the combined group (3.65 g kg^{-1}), but statistically speaking there were no real differences between the two groups. An anti-fibrolytic agent (tranexamic acid) was used during peri-operative bleeding control periods for all patients except three who complained of nausea induced by the agent. Excellent surgical results were achieved for all surgeries. The pseudotumour was removed *en bloc*. After both shoulder osteophytes were removed and the right shoulder was manipulated, the abduction and flexion of both shoulders improved to around 90°. After joint arthroplasties, pain was relieved and bleeding did not recur. After synovectomy, bleeding gradually stopped and did not recur.

However, haemostatic evaluations differed. Nine surgeries had been controlled without unexpected massive bleeding or bleeding complications (excellent), and one of the two performed pseudotumour removals had expected massive bleeding with bleeding complications (good), and another performed bipolar hip arthroplasty had unexpected massive bleeding with bleeding complications (fair).

Complications

There were no thrombogenic adverse effects, only two bleeding episodes. One episode was re-bleeding on day 9 after the operation. This bleeding was controlled with a shortened bolus infusion interval from 6 to 4.5 h. The second episode was continued bleeding from the operative day, despite good haemostasis in the operation room. We continued

Table 3. Overview of dose and periods of recombinant activated factor VII for bleeding control at peri-operative periods and complications.

Case	One dose per body weight (mcg kg^{-1})	Total dose per body weight (g kg^{-1})	Total infusion periods (days)	Bolus infusion periods (days)	Continuous infusion periods (days)	Tranexamic acid (days)	Complications
1	103.4	9.9	19	19	0	18	Bleeding
2-1	117.6	13.9	22	22	0	21	
3-1	120.0	8.5	12	12	0	13	
4-1	139.5	5.3	7	7	0	2	Nausea
5	125.0	4.5	8	8	0	7	
3-2	120.0	8.4	12	12	0	14	
6	106.7	3.1	6	6	0	6	
4-2	102.1	3.5	5	2	3	2	Nausea
2-2	120.0	2.4	5	2	3	2	Nausea
7-1	95.2	3.8	7	1	6	8	
7-2	100.0	4.9	9	2	7	8	Bleeding

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to administer rFVIIa by bolus injection, 15 times at 2-h intervals, until the bleeding started to ooze and then we changed to continuous infusion. The bleeding stopped on day 3 after the operation.

Discussion

There are two major limitations to performing surgery for haemophilia patients in Japan. First, general insurance in Japan does not permit chemical or radioactive synoviorthesis for any arthropathies or synovitis. The clinical results show that these synoviorthesis procedures are safe and effective. Internationally, these procedures are first-line therapies for haemophilic synovitis, especially for inhibitor patients. However, as we are not able to choose these procedures, arthroscopic synovectomy is the first-line therapy for haemophilic synovitis in Japan. This is the reason why we have many arthroscopic synovectomies for haemophilia with inhibitors. Another limitation concerns the use of aPCC. Until the end of March 2008, we were not permitted to use aPCC over 3 days serially and we were required to change from aPCC to other concentrates on the fourth day even if aPCC had been effective. Most of the cases in this report were performed before April 2008 and they were controlled using rFVIIa.

Orthopaedic surgery had been impossible for haemophilia with inhibitors for the past two decades. The development of bypassing agents, such as aPCC and rFVIIa made orthopaedic surgery possible, and the results of the first operation using rFVIIa was published in 1988 [7]. After this case report, orthopaedic surgery started to be performed internationally for haemophilia with inhibitors, but as an elective surgery performed only by an expert medical haemophilia team. Most of the surgical results were of minor surgeries and major surgical results were mainly reported as case reports. In terms of large major orthopaedic surgical results for haemophilia with inhibitors under rFVIIa cover, Obergfell *et al.* [4] reviewed the orthopaedic surgical results published up until 2006. According to this review, the surgical results of two to six major orthopaedic surgeries were reported among five articles from single institutes. After 2006, Harberman *et al.* [8] reported the surgical results of six surgeries, which was the largest study from a single institute. However, some multicentre studies had reported 6–18 major orthopaedic surgical results [5,9,10]. This report has 11 major orthopaedic surgical results and is the largest study from a single institute.

It is important for bleeding control using rFVIIa to increase plasma rFVIIa levels. Hoffman *et al.* [11] concluded that the doses of rFVIIa required for efficacy produce plasma levels that are several orders of magnitude greater than the *Kd* for binding of FVIIa to tissue factor, and a platelet surface mechanism is better able to explain the clinical efficacy of high-dose FVIIa therapy. In a report that included the experiences of seven European haemophilia centres, it was concluded that high doses of rFVIIa are safe and effective for the treatment and prevention of bleeding [12]. Also Salaj *et al.* [13] concluded that initial high doses of rFVIIa were associated with a decline in total rFVIIa consumption, because the rate of thrombin generation is important for fibrin clots that form in the presence of high amounts of rFVIIa and are more resistant to proteolysis, which might be of great importance in the context of joint inflammation where elevated enzyme levels exacerbate proteolysis and the destruction of synovium, cartilage and bone. In two recently published papers in which the sole aim was to establish a consensus protocol, bolus infusion was recommended as routine haemostasis therapy for haemophilia with inhibitors and continuous infusion was not recommended because more clinical studies are required for this mode of administration [6,14]. Giangrade [14] recommended that 120–180 mcg kg^{-1} be injected at the start of surgery, just prior to the first incision. Then, follow-up doses of 90 mcg kg^{-1} are given at 2–6-h intervals during the peri-operative periods.

In our experiences, the first bolus infusion was given before general anaesthesia and the second bolus infusion was given just before the first incision. The interval between the first and second infusions was shorter than 2 h. This administration mode achieved higher plasma rFVIIa levels without changing the dose and prevented bleeding incidents for unconscious patients.

However, rFVIIa has a short half-life and requires frequent administrations to keep the plasma levels of FVIIa above that required to maintain haemostasis for haemophilia with inhibitors. Therefore, there were some reports that already confirmed the stability and microbiological safety *in vitro* and *in vivo* of rFVIIa after being reconstituted [15–17]; also, the good surgical results noted in the clinical reports [15,18–20] were increased by the continuous infusion of rFVIIa as an economical mode of administration. Continuous infusion was started after initial bolus infusion. The bolus dose range was from 90 to 104 mcg kg^{-1}. The continuous infusion rate range at the start was from 16.5 to 50 mcg kg^{-1} h^{-1}, and the duration range was from 1 to 26 days. In terms of the continuous infusion rate, Schulman [15] concluded that it was possible to use 10 IU mL^{-1} as a

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maintenance level for most situations provided that precautions are taken to avoid the risk factors for hemorrhagic complications. This 10 IU mL^{-1} was calculated to be 660 IU kg^{-1} h^{-1} or 13.2 mcg kg^{-1} h^{-1}. In all the reports, the continuous infusion rates were more than 13.2 mcg kg^{-1} h^{-1} and achieved good haemostasis, except one. This continuous infusion rate at the start was 16.5 mcg kg^{-1} h^{-1}. To achieve good haemostasis during surgery and just after operation, the rFVIIa plasma levels must be higher than 10 IU mL^{-1} as a maintenance level.

Furthermore, we have another administration mode that is a combination of bolus and continuous infusion and had already been reported as continuous infusion [21–24], but here we refer to this mode as combination infusion because it consists of several bolus infusions as opposed to one bolus infusion prior to continuous infusion [18,20,25]. As for continuous infusion, Ludlam *et al.* [18] reported nine major orthopaedic surgeries in which rFVIIa was infused at a rate of 50 mcg kg^{-1} h^{-1} for 7–20 days after an initial preoperative bolus of 90 mcg kg^{-1}. Postoperatively, there were bleeds in six patients. Santagostino *et al.* [20] reported 11 major orthopaedic surgeries in which rFVIIa was infused at a rate of 20 mcg kg^{-1} h^{-1} for 3–14 days after an initial preoperative bolus of 90–135 mcg kg^{-1}. Postoperatively, there were bleeds in two patients. Smith *et al.* [25] also reported eight elective surgeries in which rFVIIa was infused at a rate of 16.5 mcg kg^{-1} h^{-1} for 1–26 days after an initial preoperative bolus of 90 mcg kg^{-1}, and they concluded that the 16.5 mcg kg^{-1} h^{-1} infusion rate reliably achieves plasma FVII activity levels of 10 IU mL^{-1}, but this level does not provide reliable haemostasis. However, for case studies related to the administration mode we referred to as combination infusion, Tagariello *et al.* [24] reported two cases in which rFVIIa was infused at a rate of 11–42 mcg kg^{-1} h^{-1} for 12–29 days after bolus infusion of 120 mcg kg^{-1} for 2–3 days. In both cases, bleeding complications occurred on days 8 and 14 after surgery, respectively. He also reported another successful case in which rFVIIa was infused five times at 2-h intervals prior to continuous infusion [23]. Pepez *et al.* [22] reported one case of a total hip arthroplasty in which rFVIIa was infused at a rate of 7–15 mcg kg^{-1} h^{-1} for 12 days after bolus infusion of 90–150 mcg kg^{-1} for 5 days, and no bleeding complications occurred. Lorenzo *et al.* [21] reported one case of an open evacuation of a large knee haemarthrosis in which rFVIIa was infused at a rate of 20 mcg kg^{-1} h^{-1} for 7 days after bolus infusion of 120 mcg kg^{-1} for 3 days, and they concluded that FVIIa plasma levels of 6–10 IU mL^{-1} were safe and effective at preventing postoperative haemorrhaging in this patient.

Based on published articles, we thought that the continuous infusion rate of 16.5 mcg kg^{-1} h^{-1}, which reliably achieves plasma FVII activity levels of 10 IU mL^{-1}, is good enough to maintain a haemostatic condition, but not to achieve good haemostasis. A higher rate of continuous infusion might achieve good haemostasis, however, two recent reports [6,14] concluded that bolus infusion is more reliable to achieve good haemostasis than continuous infusion. We believe it is most important for peri-operative haemostasis to achieve a good haemostatic condition just after surgery, and also believe that the administration of bolus infusion during surgery is more effective at stopping bleeding than continuous infusion. However, theoretically the total dose of bolus infusion administered during the peri-operative period is more than that of continuous infusion, so from an economical point of view it is also important to change from bolus infusion to continuous infusion once all bleeding has stopped. We recommend the administration of the referred to combination infusion that consists of two parts: a bolus infusion (i.e. several bolus infusions) to achieve good haemostasis and a continuous infusion to maintain a good haemostatic condition. We also believe that this combination infusion mode is the most effective and economical mode to administer.

We have performed numerous major orthopaedic surgeries for haemophilia patients with inhibitors and have achieved good haemostasis. Based on those results, we have concluded that the combination of bolus and continuous infusions of rFVIIa is safe and effective, and more convenient to administer than simple bolus infusion therapy to achieve haemostasis at peri-operative periods. In addition, our data also concurs with the data of several previous reports which showed that orthopaedic surgery for haemophilia patients with inhibitors by means of rFVIIa is safe and effective.

Disclosures

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

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原　　著

HIV 感染血液凝固異常症における AIDS 指標疾患の報告数について：血液凝固異常症全国調査に基づく集計

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目的：本邦の HIV 感染血液凝固異常症において，1997 年から 2008 年までの AIDS 指標疾患の状況を掌握すること。

対象および方法：1997 年から 2008 年までの期間について AIDS 指標疾患の報告数を調査年毎に集計した。複数の指標疾患が同時に報告されていた場合も疾患毎に独立した合計数を求めた。

結果：最も頻繁に報告されたのはカンジダ症で，死亡時に報告されたものが合計 17 例，各調査期間の生存例で報告されたものが合計 94 例であった。次いでニューモシスティス肺炎（死亡 14 例，調査時生存 53 例），HIV 消耗性症候群（死亡 22 例，調査時生存 23 例）の順であった。この他で報告数が多い疾患は，サイトメガロウイルス感染症，反復性肺炎，非結核性抗酸菌症，進行性多巣性白質脳症であった。AIDS 指標疾患の年次報告総数は，1997 年から 2002 年まで減少し，2003 年以後は年間合計 20 例未満に止まっていた。死亡時に重篤な肝疾患があった報告は累積 167 例となり，この中で肝疾患の原因が HCV 感染と考えられるものが 140 例（84%）であった。

結論：HIV 感染血液凝固異常症においては AIDS 指標疾患の新たな発症は少数で，死亡時に AIDS 指標疾患を有する例も少なくなっている。一方，HCV 感染が原因と考えられる重篤な肝疾患による死亡数は減少傾向を示しておらず，HCV 感染に対する治療を積極的に進める必要がある。

キーワード：エイズ指標疾患，血液凝固異常症，血友病，全国調査，HCV

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1. はじめに

血液凝固異常症全国調査による報告では，2008 年 5 月 31 日現在で生存中の HIV 感染血液凝固異常症の総数は 796 例で，そのうち AIDS 発症例は 166 例となっている[1)]。しかし，この AIDS 発症者数には治療によって症状が消失した例や，検査所見が改善した例も含まれており，報告の時点で 23 の AIDS 指標疾患のいずれかを有している例は少数である。本稿では血友病を中心とする血液凝固異常症と HIV 感染に関する全国的な調査が，現在のような形式に整えられた 1997 年から，最新の調査である 2008 年の調査までに報告された AIDS 指標疾患の報告数の経時的な変化をまとめ，死亡報告中の該当数も含めて報告する。

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2. 方　　法

1997 年度から 2008 年度までの血液凝固異常症の全国的な調査に報告された，HIV 感染血液凝固異常症（血友病，von Willebrand 病，類縁疾患）における AIDS 指標疾患について集計した。

当該期間の血液凝固異常症と HIV 感染に関する全国的な調査は，1997 年度から 1999 年度までは厚生労働省の「HIV 感染者発症予防・治療に関する研究班」[2)]，2000 年度については同省の「HIV 研究の評価に関する研究班」内の分担研究[3)]による調査であったので，それらの報告による集計数を用いた。

2001 年度以後の調査については現在の血液凝固異常症全国調査によって行われているので，その集積データを用