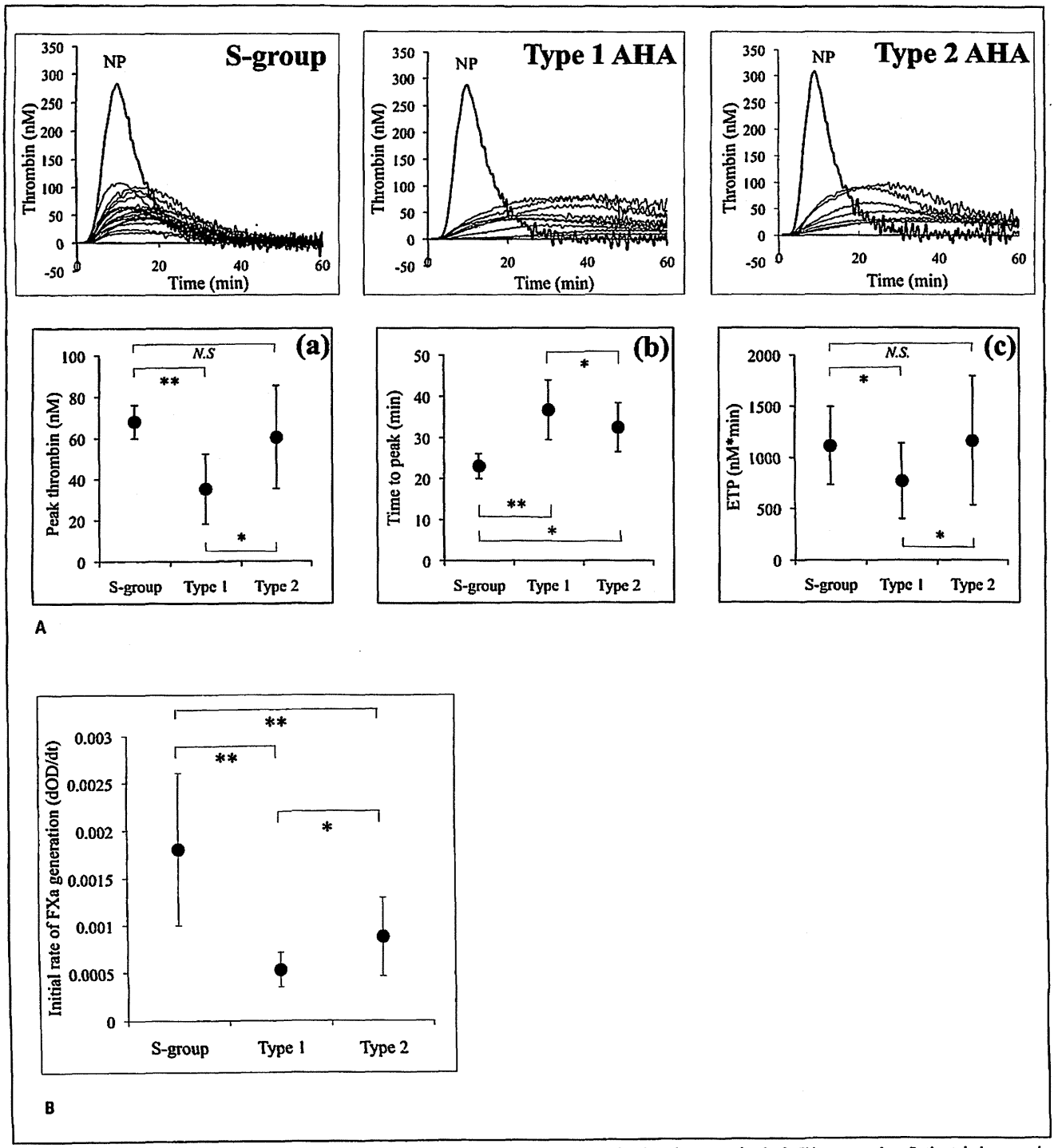


Figure 2: TGT and endogenous intrinsic FXa generation on patient's plasmas in the S-group, type 1, and type 2 AHA. A) TGT-assay; Upper panels: Patients' plasmas obtained from the S-group, type 1 AHA, and type 2 AHA were preincubated with TF (0.5 pM), PL (4 μM) and ellagic acid (0.3 μM), followed by the addition of $CaCl_2$. Thrombin generation was measured as described in *Methods*, and representative TGT curves are illustrated. NP; control normal plasma. Lower panels: The parameters of peak thrombin (a), time to peak (b), and ETP (c) were obtained from the TGT data shown in upper panels. B) Endogenous intrinsic FXa generation; Patients' plasmas obtained from S-group, type 1, and type 2 AHA were preincubated with FIXa/FX/PL mixture in the presence of hirudin, followed by the addition of $CaCl_2$ as described in *Methods*. FXa was measured using commercial reagents. The initial velocity rates of endogenous FXa generation are illustrated. In all instances, results are shown as mean ± SD from at least five separate experiments. The value of FVIII:C 1.0 IU/dl as a reference value was 5.04 ± 0.20 $x10^{-3}$. *p<0.05, **p<0.01, NS; no significance.

~2.0-fold (121 ± 44/61 ± 8 seconds, p<0.05; *panel* a), compared to those in the M-group, and both |min1| and |min2| values were significantly decreased by ~2.1-fold, (1.1 ± 0.5/2.3 ± 0.3, p<0.01; *panel* b) and by ~3.1-fold (0.06 ± 0.03/0.19 ± 0.04, p<0.01; *panel* c), respectively. These results demonstrated that blood coagulation in type 2 was markedly more defective than in the M-group, despite similar FVIII:C levels (2.1 ± 0.9/2.0 ± 1.9 IU/dl, respectively). The findings were in keeping with the more severe haemorrhagic symptoms observed in type 2 relative to the M-group of patients.

Comparisons of coagulation function in severe HA (S-group) and AHA

More severe haemorrhagic symptoms are evident in the AHA patients compared to those in the S-group (FVIII:C<0.2%). These clinical differences were examined, therefore, using the TGT in these patients. Representative thrombograms from the S-group, type 1, and type 2 are illustrated in ▶Figure 2A (*upper panels*). The derived parameters are shown in the *lower panels*. The levels of peak thrombin and ETP in type 1 were markedly decreased by ~2-fold (type 1/S-group: 35.2 ± 14.1/68.0 ± 8.2 nM, p<0.01, *panel* a) and by ~1.5-fold (770 ± 310/1,115 ± 381 nM, p<0.05, *panel* c), respectively. The time to peak in type 1 was significantly prolonged by ~1.6-fold (36.7 ± 6.5/23.0 ± 3.0 min, p<0.01, *panel* b), compared to those in S-group. Similarly, in type 2, the time to peak was significantly delayed compared to that in S-group (32.5 ± 6.0/23.0 ± 3.0 min, p<0.05, *panel* b). These findings again provided strong evidence that the more serious clinical symptoms in AHA were related to the differences in global coagulation profiles, even though the FVIII:C in AHA were similar or slightly higher level than those in S-group. Surprisingly, thrombin generation in type 1 was moderately, but significantly more defective than in type 2 (p<0.05). It appeared, therefore, that coagulation function in the three groups was depressed in the order type 1, type 2, S-group.

Intrinsic FXa generation, corresponding to the upstream process of thrombin generation, was further examined to clarify the mechanism(s) of excessively defective thrombin generation in AHA. Plasma samples from each of the three groups were incubated with FIXa/FX/PL mixtures in the presence of hirudin (to eliminate thrombin reactions). $CaCl_2$ was added and endogenous intrinsic FXa generation was measured using the chromogenic assay. The initial rate of FXa generation was decreased in the order type 1, type 2, S-group (0.53 ± 0.18/0.88 ± 0.41/1.81 ± 0.78 x10^{-3}) with significant differences (▶Fig. 2B). These results were consistent with those obtained in the TGT, and further suggested that the discrepancies in coagulation function between AHA and S-group HA could be attributed to a significant decrease in the expression of intrinsic tenase complex activity (FVIIIa/FIXa/FX/PL).

Properties of anti-FVIII autoAbs in AHA

To further investigate the mechanism(s) by which the coagulation function in AHA was more defective than in the S-group, anti-FVIII autoAbs purified from AHA plasmas were characterised. FVIII levels and the basic properties of these autoAbs are summarised in ▶Table 1. Other coagulation factor activities in all cases were within the normal range (data not shown). SDS-PAGE and Western blotting using purified coagulation proteins revealed that all autoAbs reacted with FVIII alone. In particular, they all strongly reacted with the C2 domain, although some additionally reacted very faintly with the A2 domain.

The C2 domain is associated with interactions with VWF and PL (33). We examined, therefore, the effects of anti-C2 autoAbs on FVIII binding to VWF and PL in ELISA. In all type 1 cases examined the antibodies dose-dependently inhibited FVIII binding to VWF (by 64–87%) and PL (by 60–79%) at the maximum concentration of 50 μg/ml (▶Table 2), and the inhibitory effects were dose-dependent (data not shown). In contrast, in all type 2 cases the antibodies did not affect binding. Insufficient amounts of purified $F(ab')_2$ were obtained from some type 1 cases (cases 7–9) and type 2 cases (cases 16–17), however, and these individuals could not be investigated.

Different effects of anti-C2 autoAbs on thrombin-catalysed FVIII reactions

The conversion of FVIII to FVIIIa by thrombin is essential for the expression of intrinsic tenase activity (5), and one particular FVIII binding-region has been located within the C2 domain (34). We examined, therefore, the effects of anti-C2 autoAbs on thrombin-catalysed FVIII activation. FVIII (0.05 nM) was preincubated with varying amounts of AHA autoAbs. After incubation with thrombin for 1 min, the reaction was stopped by the addition of hirudin, and the reactant mixtures were diluted to completely exclude the inhibitory effects of autoAbs. FXa generation was initiated by the addition of FIXa (1 nM) and FX (150 nM) (▶Fig. 3A, *upper panel*). Results are summarised in ▶Table 2. All type 2 antibodies (50 μg/ml) decreased the peak levels of thrombin-mediated FVIII activation by 66–94%, and the inhibitory effects were dose-dependent. Type 1 autoAbs little affected these reactions (by <5%), however. In these experiments, the presence of anti-C2 autoAbs may have interfered with FXa generation and indirectly moderated thrombin-catalysed FVIII activation. To investigate this, therefore, we examined direct thrombin-catalysed FVIII cleavage in the presence of anti-C2 autoAbs. Proteolytic cleavage at Arg^{372} and Arg^{1689} is essential for generating FVIIIa activity (5). FVIII (10 nM) was preincubated with anti-C2 autoAbs (≤100 μg/ml), and was then activated by thrombin (5 nM), followed by SDS-PAGE and Western blotting using anti-A2 mAbJR8 (▶Fig. 3A, *lower panels*). All type 2 antibodies delayed the appearance of intact A2 during early-timed reactions (*panel* a). The inhibitory effects were dose-dependent by 61–73% (at 50 μg/ml), and were consistent with inhibition of

Table 2: Properties of anti-C2 autoAbs obtained from AHA patients.

Case	Inhibition of FVIII binding to		Inhibition of thrombin-catalysed reaction of FVIII			Inhibition of FXa-catalysed reaction of FVIII			Competition of FVIII binding to	
	VWF	PL	Activation	Cleavage (%)		Activation	Cleavage (%)		ESH4	ESH8
	(%)		(%)	Arg372	Arg1689	(%)	Arg372	Arg1689	(%)	
Type 1										
1	67	72	<5	<5	<5	n.d.	n.d.	n.d.	78	12
2	86	64	<5	<5	<5	n.d.	n.d.	n.d.	69	15
3	64	60	<5	<5	<5	n.d.	n.d.	n.d.	73	<5
4	77	63	<5	<5	<5	n.d.	n.d.	n.d.	69	<5
5	72	69	<5	<5	<5	n.d.	n.d.	n.d.	63	<5
6	87	79	<5	<5	<5	n.d.	n.d.	n.d.	84	<5
Type 2										
10	<5	<5	84	70	80	68	72	>95	<5	69
11	<5	<5	81	69	72	92	66	>95	<5	77
12	<5	<5	66*	64	35	63*	72	>95	<5	64
13	<5	<5	83	61	40	95	70	>95	14	86
14	<5	<5	73	63	68	83	79	>95	19	84
15	<5	<5	94	73	80	59	54	>95	<5	67

Reactions of anti-C2 autoAbs were examined as described in *Methods*. Data represent the inhibitory effects (%) at concentrations of 50 µg/ml for all cases except for case 12* (70 µg/ml). Insufficient amounts of $F(ab')_2$ were available from cases 7–9 (Type 1) and cases 16–17 (Type 2). n.d.: not determined.

cleavage at Arg^{372} (*panel* b). Similarly, inhibition of cleavage at Arg^{1689} (by 35–80%) was observed with all type 2 antibodies (▶Table 2). These cleavage patterns appeared to be little affected (by <5%) by type 1 antibodies, consistent with the results of FVIII activation.

FXa-catalysed FVIII activation was also investigated, as a target for inhibitory effect of anti-C2 autoAbs. It was difficult, however, to assess FVIIIa-dependent FXa generation in the presence of purified FXa as an activator of FVIII. Consequently, FVIII-dependent FIXa-catalysed FXa generation was evaluated. This assay depended on the positive-feedback mechanism(s) by which FIXa-catalysed FXa generation mediated FVIII activation. FVIII (0.05 nM) was preincubated with varying amounts of autoAbs, followed by the addition of FIXa (1 nM), FX (150 nM), PL (20 µM), and hirudin to initiate FXa generation (▶Fig. 3B). All type 2 antibodies (50 µg/ml) diminished the level of FXa generation by 59–95%, and the inhibitory effects were dose-dependent (*upper panel*). To directly examine FXa-catalysed FVIII proteolysis, FVIII (10 nM) was mixed with autoAbs (50 µg/ml) prior to incubation with FXa (0.5 nM) and PL (20 µM) (▶Fig. 3B, *lower panels*). All type 2 antibodies inhibited cleavage at Arg^{372} by 54–79% in a timed-dependent manner, and the inhibitory effects were dose-dependent (*panels* a and b). Cleavage at Arg^{1689} was also completely inhibited (by >95%) by all type 2 antibodies (▶Table 2). The inhibitory effects of type 1 antibodies could not be determined precisely, however, since these antibodies directly inhibited FVIII(a)-PL interaction.

Coagulation function in AHA-model reconstituted with FVIII/anti-C2 mAb

The inhibitory properties of anti-C2 type 1 and type 2 autoAbs obtained in the present study were similar to those reported by Meeks et al. (9, 10). To investigate whether the pivotal C2 epitopes of our autoAbs overlapped with those of anti-C2 mAbESH4 or mAbESH8, representing typical type 1 or type 2 behaviour, respectively, competitive inhibition for FVIII binding were examined. All type 1 autoAbs significantly competed with ESH4 binding to FVIII by 63–84%, but competed with ESH8 binding by <5–15%. In contrast, all type 2 autoAbs competed with ESH8 binding to FVIII by 64–86%, but competed with ESH4 binding by <5–19%. These findings indicated that anti-C2 type 1 and type 2 autoAbs contained the C2 epitopes identified in ESH4 and ESH8, respectively (▶Table 2).

We compared, therefore, the coagulation parameters in *in vitro* models of AHA, constructed with exogenous anti-C2 mAbs (ESH4 and ESH8), with those of the S-group. FVIII (10 IU/dl) was preincubated with ESH4 (80 µg/ml) or ESH8 (20 µg/ml), and residual FVIII:C was adjusted to <0.2 and ~2 IU/dl, respectively, similar to the levels in AHA patients. The mixtures were added to FVIII-deficient plasma and utilised in the TGT-assay (▶Fig. 4A and B). The time to peak in the AHA-models with ESH4 and ESH8 (48.8 ± 2.0/47.6 ± 2.4 min, $p<0.01/p<0.05$, respectively) were prolonged compared to the S-group (43.0 ± 1.6 min), reflecting decreased coagulation function in the presence of ESH4/ESH8. These findings

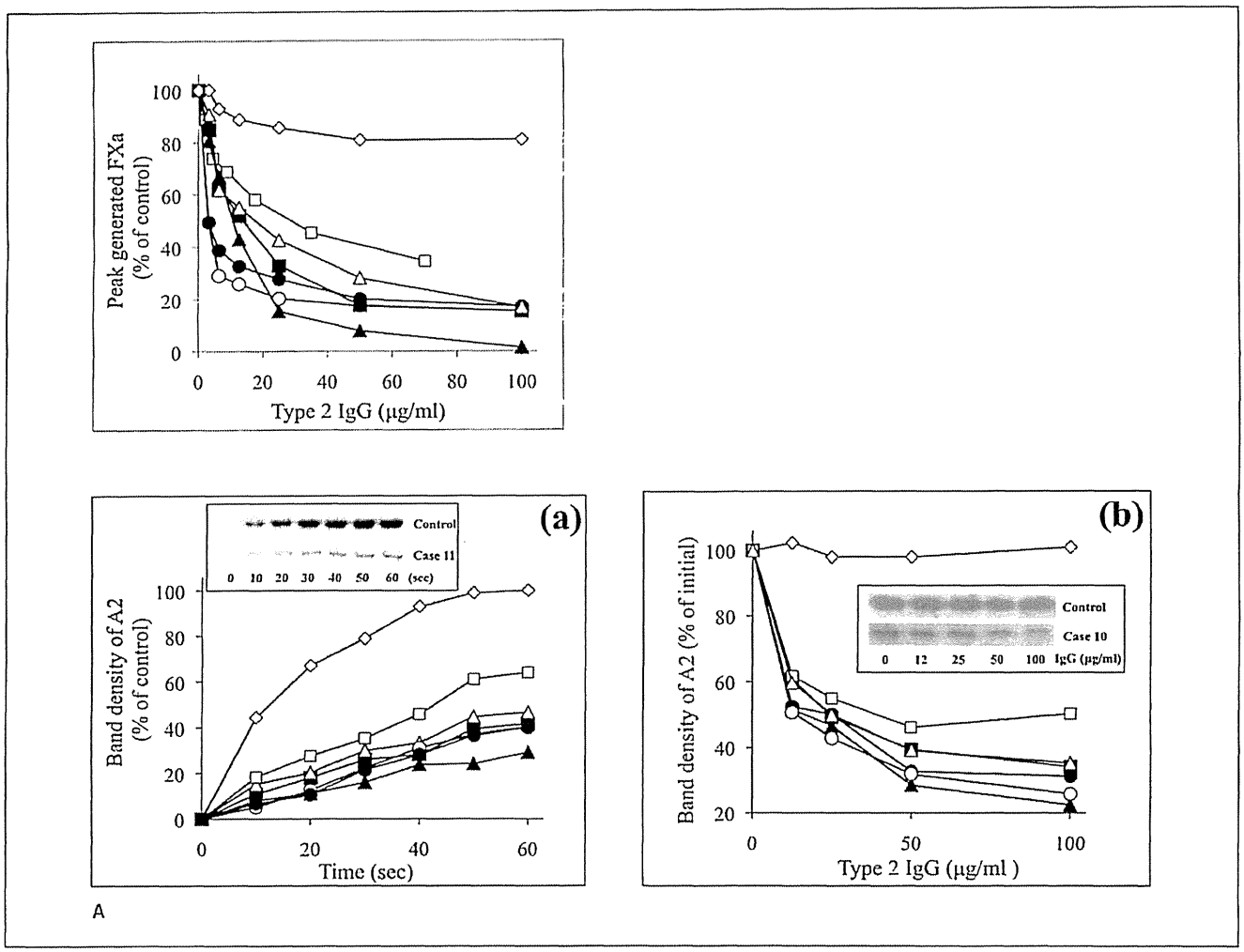
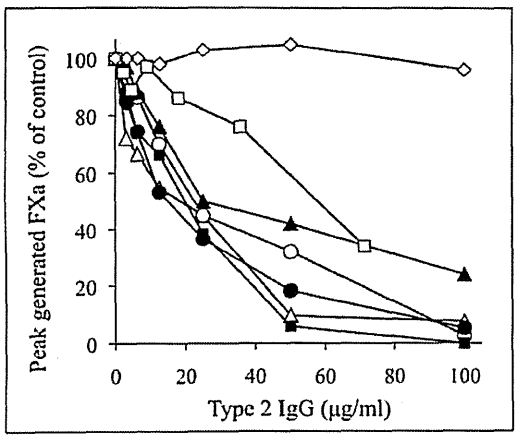
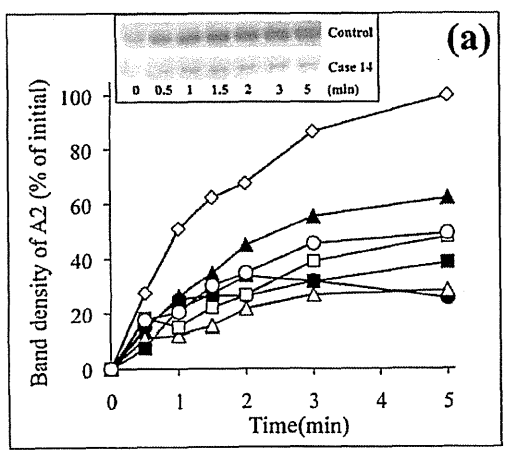
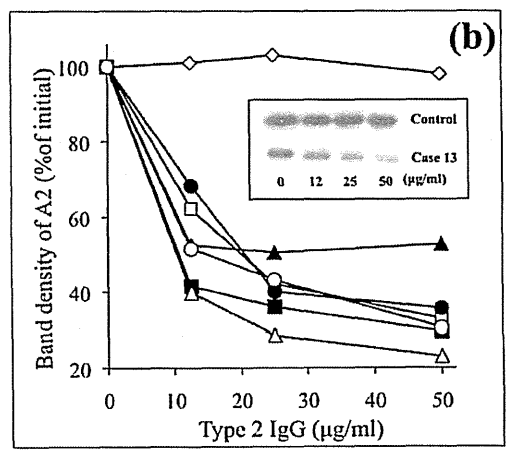


Figure 3: Effects of type 2 anti-C2 autoAbs on thrombin- or FXa-catalysed activation of FVIII. A) Thrombin reaction; Upper panel: FVIII (0.05 nM) was activated by thrombin (1 nM) for 1 min. After the addition of hirudin and dilution, FXa generation was initiated by the addition of FIXa (1 nM), FX (150 nM), and PL (20 μM). Various concentrations of type 2 autoAbs were preincubated with FVIII prior to adding thrombin, followed by adding hirudin to terminate the thrombin reaction. The rate of FXa generation without anti-C2 autoAb was regarded as 100%. In all instances, results are shown as mean from at least five separate experiments. Lower panels: (a) FVIII (10 nM) was mixed with type 2 autoAbs (100 μg/ml) for 1 h, followed by incubation with thrombin (5 nM) for the indicated times. Samples were run on 8% gel followed by Western blotting using anti-A2 mAbJR8. Band density of A2 at 1 min after adding thrombin with normal $F(ab')_2$ was regarded as 100%. (b) FVIII (10 nM) was mixed with various concentrations of type 2 autoAbs for 1 h, followed by incubation with thrombin (5 nM) for 1 min. Samples were run on 8% gel followed by Western blotting using anti-A2 mAb. Band density of A2 after adding thrombin in the absence of type 2 autoAbs was regarded as 100%. B) FXa reaction; Upper panel: FVIII (0.05 nM) was incubated with various concentrations of type 2 autoAbs for 1 h. FXa generation was initiated by the addition of FIXa (1 nM), FX (150 nM), PL (20 μM) in the presence of hirudin for 30 min. The rate of endogenous intrinsic FXa generation in the absence of autoAb was regarded as 100%. In all instances, results are shown as mean from at least five separate experiments. Lower panels: (a) FVIII (10 nM) was mixed with type 2 autoAbs (50 μg/ml) for 1 h, followed by incubation with FXa (0.5 nM) and PL (20 μM) for the indicated times. Samples were run on 8% gel followed by Western blotting using anti-A2 mAb. Band density of A2 at 5 min after FXa incubation with normal $F(ab')_2$ was regarded as 100%. (b) FVIII (10 nM) was mixed with various concentrations of type 2 autoAbs for 1 h, followed by incubation with FXa (0.5 nM) and PL (20 μM) for 5 min. Samples were run on 8% gel followed by Western blotting using anti-A2 mAb. Band density of A2 by FXa in the absence autoAbs was regarded as 100%. The symbols used are: ○; case 10, ●; case 11, □; case 12, ■; case 13, △; case 14, ▲; case 15, ◊; normal $F(ab')_2$.







B

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

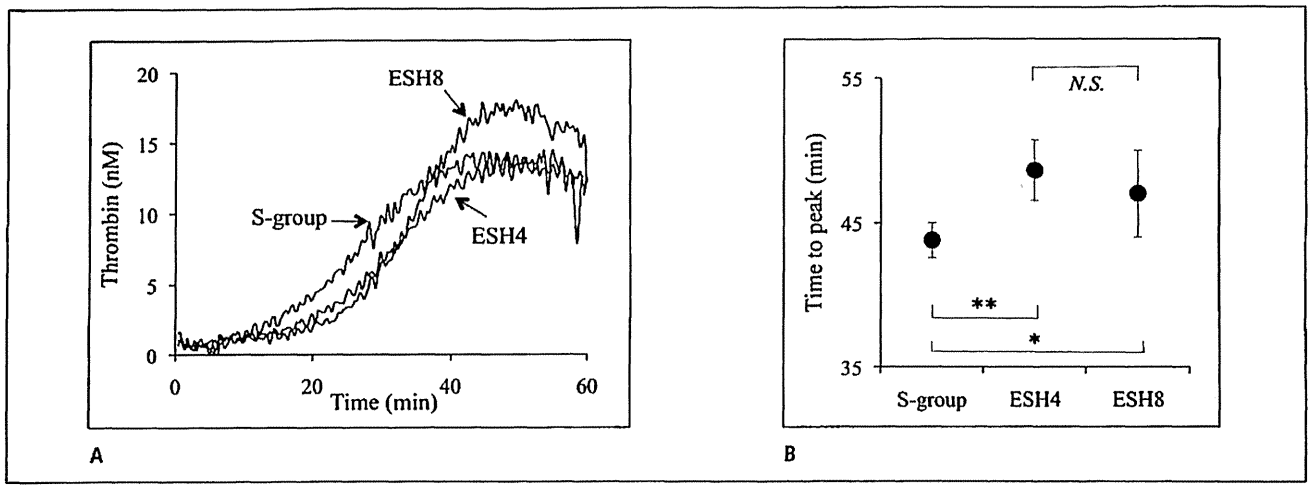


Figure 4: Coagulation function in *in vitro* AHA-models reconstituted with FVIII and anti-C2 mAb. A) FVIII (0.1 nM) was mixed with anti-C2 mAb ESH4 (80 μg/ml) or mAbESH8 (20 μg/ml) for 1 h prior to incubation with FVIII-deficient plasma. Samples were mixed with TF (0.5 pM), PL (60 μM), and ellagic acid (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.

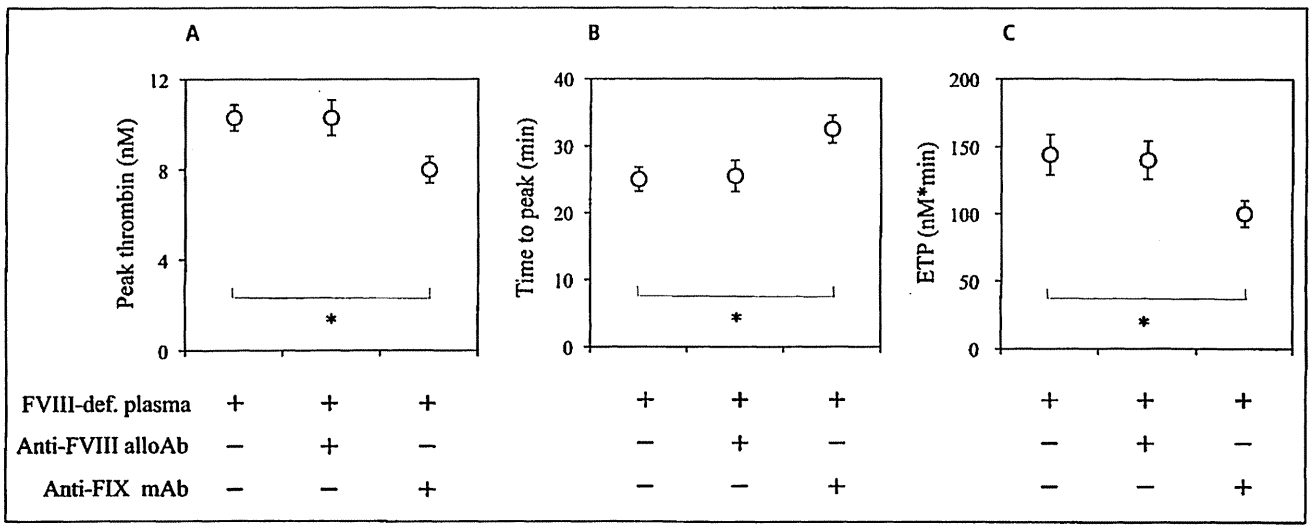


Figure 5: Effect of anti-FIX mAb on the thrombin generation in FVIII-deficient plasmas. FVIII-deficient plasma was preincubated with or without anti-C2 alloAb (10 BU/ml) for 1 h, and was reacted with or without anti-FIX Ab (10 BU/ml) for 1 h. These samples were reacted with TF (0.5 pM), PL (60 μM), and 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

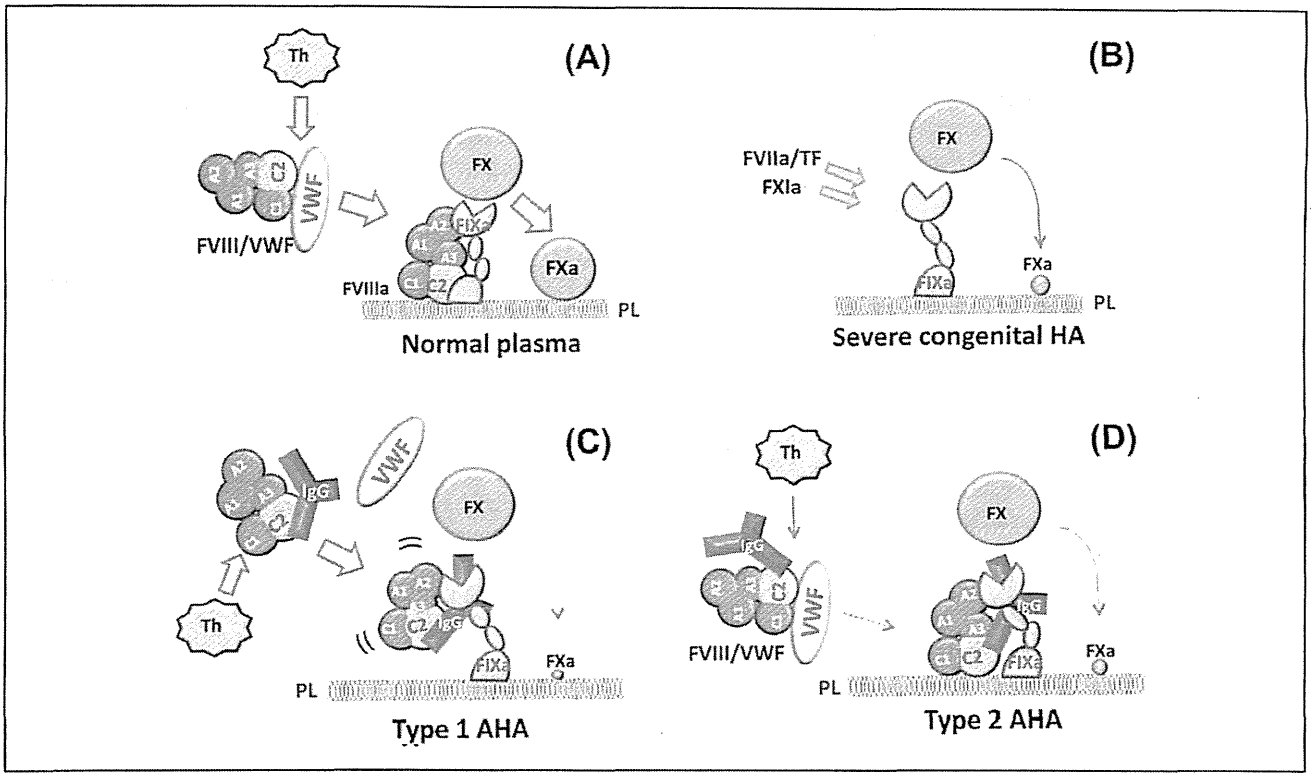


Figure 6: A putative coagulation mechanism for the intrinsic tenase complex in patients from the S-group, anti-C2 type 1, and type 2 AHA. In normal plasmas, free FVIIIa is generated from FVIII/VWF by thrombin, followed by FVIIIa/FIXa-dependent FX activation on PL micelles (A). In severe HA, FIXa alone 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.

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Conflict of interest

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ORIGINAL ARTICLE *Laboratory science*

Results of clot waveform analysis and thrombin generation test for a plasma-derived factor VIIa and X mixture (MC710) in haemophilia patients with inhibitors—phase I trial: 2nd report

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Summary. We reported the results of a clinical pharmacological study of MC710 (a mixture of plasma-derived FVIIa and FX) in haemophilia patients with inhibitors during a non-haemorrhagic state. This report provides the results of a clot waveform analysis (CWA) and thrombin generation test (TGT) using blood samples obtained in this study. CWA and TGT were conducted using blood samples obtained from a pharmacokinetic and pharmacodynamic study in which MC710 (five dose rates: 20, 40, 80, 100 and 120 μg kg^{-1}) was compared with NovoSeven (120 μg kg^{-1}) and FEIBA (two dose rates: 50 and 75 U kg^{-1}) as control drugs in 11 haemophilia patients with inhibitors without haemorrhagic symptoms. CWA showed that MC710 provided significantly greater improvement than the control drugs in activated partial thromboplastin time (APTT) at 80 μg kg^{-1}; maximum clot velocity and maximum clot acceleration were more enhanced by MC710 than by control drugs. TGT revealed that MC710 significantly shortened the initiation time of thrombin generation in comparison to FEIBA and induced greater thrombin generation potency than NovoSeven. It was not clear whether or not MC710 caused significant dose-dependent changes in the two measurements; however, differences between MC710 and the control drugs were clarified. MC710 was confirmed to have superior coagulation activity and thrombin productivity and is expected to have superior bypassing activity.

Keywords: bypassing agent, clot waveform analysis, factor VIIa, factor X, haemophiliacs with inhibitors, thrombin generation test

Introduction

Bleeding events in haemophilia patients with inhibitors (particularly patients with high titres and high-responder types) are controlled predominantly with haemostatic bypassing agents, recombinant activated factor VII (rFVIIa; NovoSeven, Novo Nordisk A/S,

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Bagsværd, Denmark) and activated prothrombin complex concentrate (APCC; FEIBA, Baxter International Inc., Deerfield, IL, USA), and sequential therapy with both agents [1]. However, these agents do not always achieve haemostasis; therefore, the development of a new agent or method of administration for more effective haemostasis is required. MC710 is a novel bypassing agent containing activated factor VII (FVIIa) and factor X (FX) highly purified from human plasma by affinity chromatography using anti-FVII and anti-FX monoclonal antibodies and mixed at a protein weight ratio of 1:10 [2]. MC710 is designed to administer FVIIa and its substrate FX concomitantly. Thus, MC710 is expected to be more potent than agents containing FVIIa alone, and longer acting due to the long half-life of FX [3].

We conducted a clinical pharmacological study (Phase I trial) of MC710 in haemophilia patients with inhibitors to evaluate its effects on pharmacokinetic (PK) and pharmacodynamic (PD) parameters and safety [4]. A clot waveform analysis (CWA) and thrombin generation test (TGT) were conducted in patients treated with MC710, NovoSeven and FEIBA in addition to measurements of conventional activated partial thromboplastin time (APTT) and prothrombin time (PT). CWA provides a chronological coagulation profile through real-time monitoring of changes in turbidity during coagulation and, in addition to measuring clotting time, calculates maximum clot velocity and maximum clot acceleration after the initiation of coagulation by first and second differentiation [5]. TGT monitors real-time changes in fluorescence intensity that chronologically increases during the thrombin generation process and quantitatively analyses thrombin production using parameters obtained in a thrombogram [6].

This report provides an analysis of the dose dependency of CWA and TGT in plasma samples obtained from a clinical pharmacological study of MC710 given as single doses at five dose rates in haemophilia patients with inhibitors. The changes in parameters were compared with those generated by control drugs (NovoSeven and FEIBA).

Materials and methods

Study design and drugs

This study was designed as a multi-centre, open-labelled, non-randomized, active controlled crossover clinical pharmacological study for Japanese male haemophilia patients with inhibitors. MC710 was administered intravenously as a single dose (20, 40, 80, 100, or 120 $\mu g\ kg^{-1}$) to male congenital haemophilia patients with inhibitors, but without haemorrhage with re-administration at different dose rates allowed. All subjects provided written informed consent as approved by the institutional review board of each participating institute. Prior to the administration of MC710, a single clinical dose of NovoSeven and/or FEIBA was administered intravenously as a control. The dose of NovoSeven was set at 120 $\mu g\ kg^{-1}$, whereas the FEIBA dose was set at the usual clinical dose for each patient, 50 or 75 U kg^{-1} [4].

Blood samples were drawn using evacuated blood collection tubes containing sodium citrate preadministration of the agents and 10 min, 2, 6, 12 and 24 h postadministration of MC710, 10 min, 2 and 6 h postadministration of NovoSeven and 10 min, 2, 6 and 24 h postadministration of FEIBA.

MC710, NovoSeven and FEIBA were provided by KAKETSUKEN (Kumamoto, Japan), Novo Nordisk A/S and Baxter International Inc. respectively. Normal human plasma (NHP; FACR, Georgeking, KS, USA) was used as a control.

Clot waveform analysis

Clot waveform analysis was performed by the standard method for determining APPT using a MDA® II automated coagulation analyser (bioMérieux, Marcy l'Etoile, France) [5]. After 50 μL of APTT reagent (ellagic acid and phospholipid (PL) solution, Baxter International Inc.) and 50 μL of $CaCl_2$ solution (final concentration: 5 mM) were added to 50 μL of patient plasma, the APTT clot waveform was monitored in real-time using an MDA® II. In this study, the maximum clot velocity (|Min1|) and the maximum clot acceleration (|Min2|) defined the minimum value of the first derivative of transmittance change (dT/dt) and the minimum value of the second derivative of transmittance change (d^2T/dt^2) respectively. The assay results were assured by confirming the parameter values of normal, FVIII-depleted, or FIX-depleted plasma in every measurement laid within 20% of the average values prior to the assay in six measurements ×3 days.

Thrombin generation test

Thrombin generation test was conducted as described previously [6]. Twenty microlitres of PPP-Reagent LOW (final concentration: 1 pM tissue factor (TF) and 4 μM PL, Thrombinoscope, Maastricht, Netherlands) and 20 μL of the fluorescent substrate Z-G-G-R-MCA (Calbiochem-Merck KGaA, Darmstadt, Germany) were added to 80 μL of human plasma. The change in fluorescence intensity caused by the release of aminomethylcoumarin hydrolysed by the generated thrombin was monitored in real-time using a Fluoroskan fluorescence plate reader (Thermo Electron Corp., Waltham, MA, USA) and that was calibrated using a thrombin standard reagent (Thrombinoscope BV, Maastricht, Netherlands). The following parameters

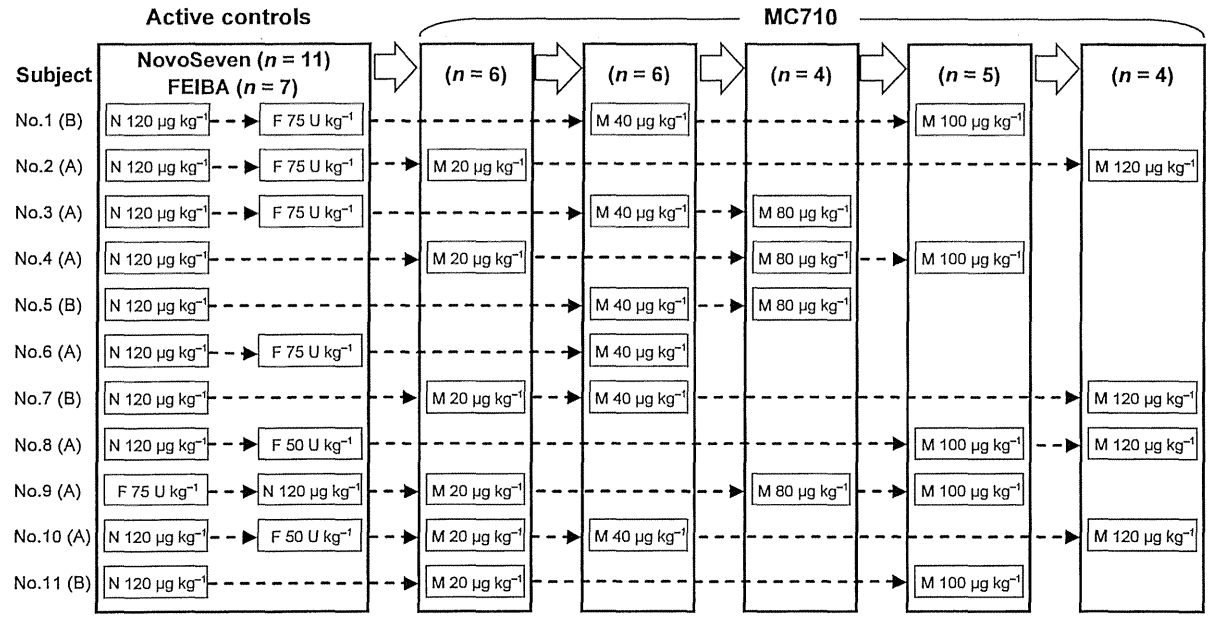

Fig. 1. Study flow chart. Study flow chart of the Phase I trial is shown in the figure. The letter within the parentheses after the subject No. indicates the type of haemophilia: (A) indicates a haemophilia A patient with inhibitors, and (B) indicates a haemophilia B patient with inhibitors. M, N and F mean MC710, NovoSeven and FEIBA respectively. Refer to the text for details.

were calculated from the profile obtained using TGT software (Thrombinoscope BV): (i) time from the beginning of measurement to the initiation of thrombin generation (Lag time); (ii) time until a peak was reached (time to peak: ttPeak); (iii) peak thrombin generation (Peak Th); and (iv) the integrated value of the area under the thrombin generation wave, reflecting the total amount of thrombin generated (endogenous thrombin potential: ETP). The assay results were assured by the same method described for CWA.

Statistical analysis

The MC710 dose-dependency of each parameter was analysed using a mixed-effects model with subject as a random effect and dose (including observation time in the parameter analysis) as a fixed effect. Differences between MC710 and the active control were analysed using a mixed-effects model with subject as a random effect and treatment and observation time as fixed effects. SAS Release 9.1 (SAS Institute Inc., Cary, NC,

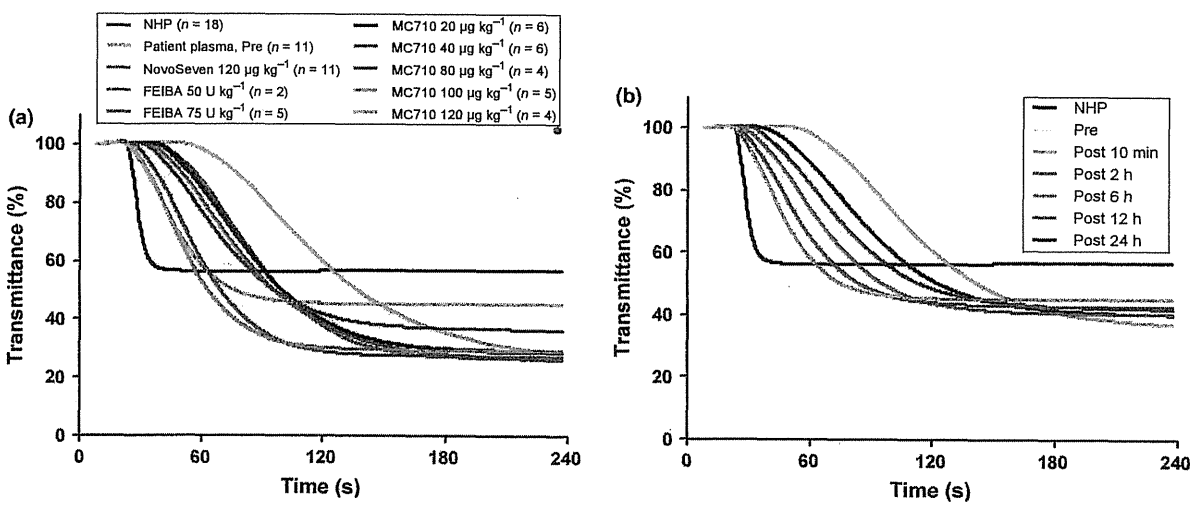

Fig. 2. APTT clot waveform profiles. Changes in mean profiles of APTT clot waveform at 10 min postadministration of control drugs and MC710 at five dose rates (a), and pre and postadministration (10 min, 2, 6, 12 and 24 h) of MC710 at 120 μg kg^{-1} (n = 4) (b) are shown. The black line indicates the mean profile for NHP (18 times repetitive measurement).

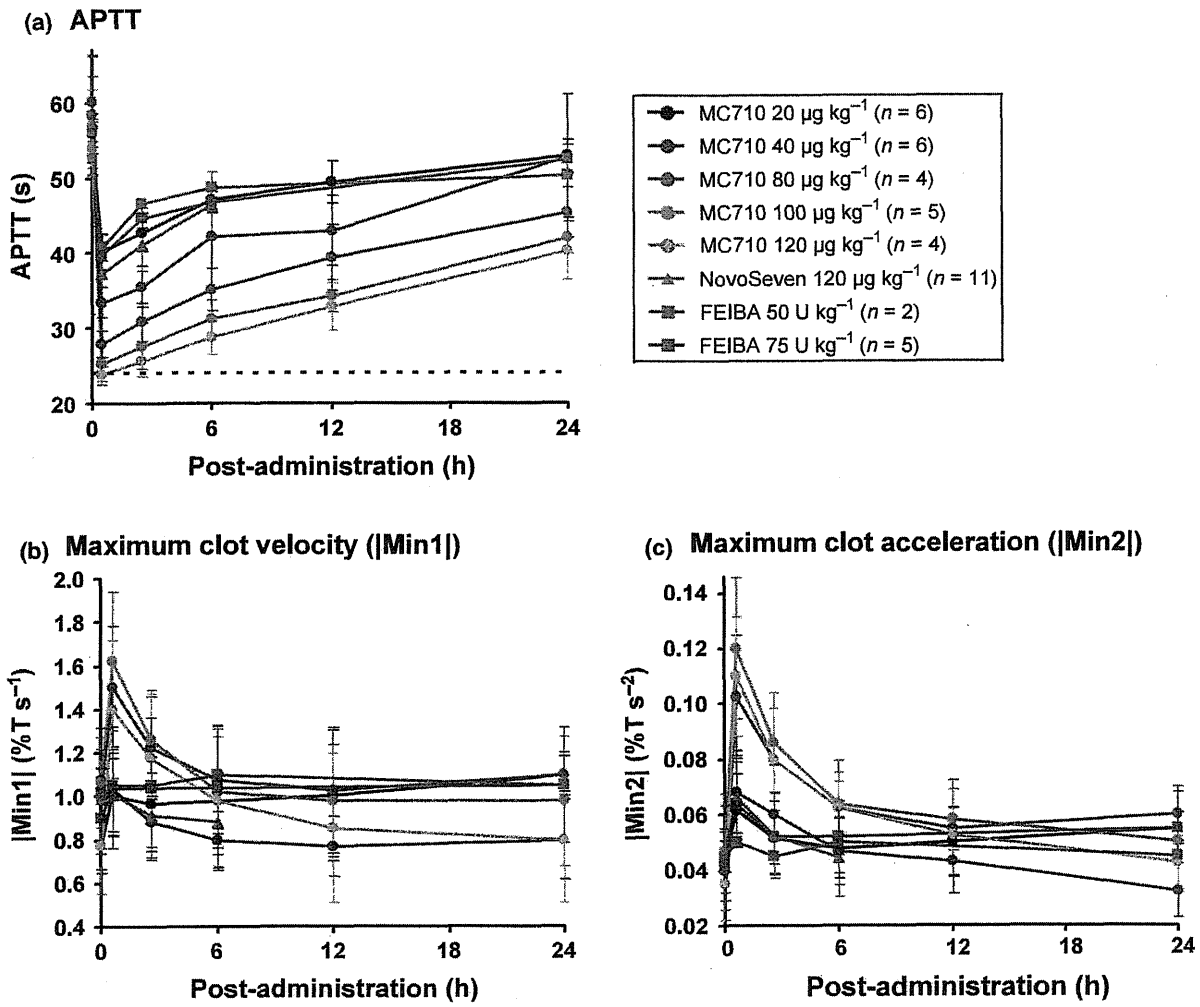

Fig. 3. Changes in CWA parameters. Time-dependent changes in CWA parameters obtained from APTT clot waveform profiles are shown. The assay was conducted using the plasma at pre and postadministration; 10 min, 2, 6, 12 and 24 h for five dose rates of MC710, 10 min, 2 and 6 h for NovoSeven, 10 min, 2, 6 and 24 h for FEIBA. (a) APTT; (b) |Min1|; (c) |Min2| (mean ± SD). The same symbols and lines are used in (a)–(c). Dashed line in (a) indicates the mean value of APTT for NHP (18 times repetitive measurement). Parameter values for NHP (mean ± SD) are APTT; 24.09 ± 0.28 s, |Min1|; 5.15 ± 0.13%T s^{-1} and |Min2|; 0.51 ± 0.01%T s^{-2}. Mean values of |Min1| and |Min2| for NHP are not denoted in (b) and (c).

USA) was used for statistical analyses. All reported *P*-values are two-tailed and not adjusted for multiple testing. $P < 0.05$ were statistically significant.

Results

Outline of the trial

The agents and doses were reported previously [4]. In brief, a total of 25 administrations doses of MC710 were given to 11 subjects (seven haemophilia A patients with inhibitors and four haemophilia B patients with inhibitors) at five dose rates after administration of active controls (Fig. 1). Subjects' mean age was 27.2 years (17–41 years) and the mean body weight was 61.3 kg (46.5–86.2 kg). The FVIII and FIX inhibitor titres immediately before administration of the investigational product ranged from 2.9 to 633 BU mL^{-1} and from 1.9 to 89.3 BU mL^{-1} respectively.

APTT clot waveform analysis

The mean clot waveform profiles for NHP and plasma obtained from patients at 10 min postadministration of each agent are shown in Fig. 2a. Transmittance of the analysis rapidly decreased with the initiation of coagulation and reached a plateau after clot formation. The mean profiles at 10 min postadministration, when the change in the waveform was greatest, showed similar transmittance reductions for 120 μg kg^{-1} NovoSeven, 50 and 75 U kg^{-1} FEIBA

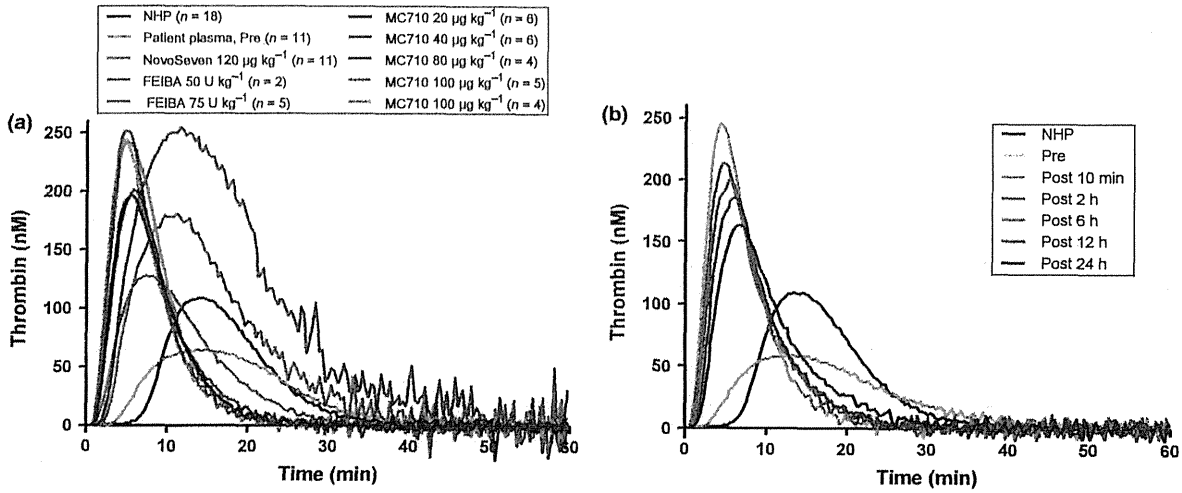

Fig. 4. Thrombin generation profiles. Changes in mean profiles of thrombin generation at 10 min postadministration of control drugs and MC710 at five dose rates (a), and mean profiles of thrombin generation pre and postadministration (10 min, 2, 6, 12 and 24 h) of MC710 at 120 μg kg^{-1} (n = 4) (b) are shown. The black line indicates the mean profile for NHP (18 times repetitive measurement).

and 20 and 40 μg kg^{-1} MC710, whereas the reductions for 80, 100 and 120 μg kg^{-1} MC710 were greater, but of a similar pattern (Fig. 2a). The profile for plasma obtained postadministration of MC710 at 120 μg kg^{-1} was closest to the NHP profile. The mean APTT clot waveform profiles for plasma obtained pre and postadministration of MC710 at 120 μg kg^{-1} showed a time-dependent change, i.e. with the most rapid decrease in transmittance being recorded 10 min postadministration followed by a gradual recovery to the preadministration profile (Fig. 2b).

In the CWA, the effect of improving prolonged APTT was significantly dependent on MC710 dose ($P < 0.001$) (Fig. 3a), consistent with previous results [4]. The effect of MC710 on APTT at doses of 80, 100 and 120 μg kg^{-1} was significantly greater than that of NovoSeven at 120 μg kg^{-1} and FEIBA at 75 U kg^{-1} (MC710 against NovoSeven: $P = 0.010$ at 80 μg kg^{-1}, $P < 0.001$ at 100 μg kg^{-1}, $P < 0.001$ at 120 μg kg^{-1}; MC710 against FEIBA: $P = 0.049$ at 80 μg kg^{-1}, $P = 0.022$ at 100 μg kg^{-1}, $P = 0.008$ at 120 μg kg^{-1}).

Both |Min1| and |Min2| were greatest 10 min postadministration of MC710 and had almost returned to preadministration levels after 12 h. The increases were not dose-dependent, but greater at ≥ 80 μg kg^{-1} MC710 than at 120 μg kg^{-1} NovoSeven or 50 or 75 U kg^{-1} FEIBA (Fig. 3b and c).

Thrombin generation test

The mean profiles of thrombin generation for NHP and for plasma obtained from patients administered each agent are shown in Fig. 4a. At 10 min postadministration, when the change in thrombin generation was greatest, there was one pattern for plasma collected postadministration of MC710 at 20 and 40 μg kg^{-1}, and another pattern for plasma collected postadministration of MC710 at 80, 100 and 120 μg kg^{-1}. In comparison with the controls, Peak Th levels were higher at all doses of MC710 than for NovoSeven at 120 μg kg^{-1} and ttPeaks were shorter at all doses of MC710 than with FEIBA at 50 or 75 U kg^{-1} (Fig. 4a). Compared to the mean thrombin generation profile preadministration, MC710 at 120 μg kg^{-1} produced the shortest time to peak generation and the highest peak. The mean profiles showed a time-dependent change, i.e. a gradual return to the preadministration profile; however, a higher peak than that obtained for NHP was maintained for up to 24 h postadministration (Fig. 4b).

In the TGT, reductions in Lag time and ttPeak were greatest 10 min postadministration and both parameters slowly returned to preadministration values (Fig. 5a and b). Similarly, enhancement of Peak Th and EPT was greatest at 10 min; the values gradually returned to preadministration levels (Fig. 5c and d). The changes in these parameters did not significantly depend on MC710 dose.

The effect of MC710 on Lag time and ttPeak was significantly greater than that of FEIBA at 75 U kg^{-1} (Lag time: MC710 against FEIBA: $P = 0.004$ at 20 μg kg^{-1}, $P = 0.017$ at 40 μg kg^{-1}, $P = 0.008$ at 80 μg kg^{-1}, $P = 0.014$ at 100 μg kg^{-1}, $P = 0.001$ at 120 μg kg^{-1}; ttPeak: MC710 against FEIBA: $P = 0.001$ at 20 μg kg^{-1}, $P = 0.001$ at 40 μg kg^{-1}, $P = 0.052$ at 80 μg kg^{-1}, $P = 0.013$ at 100 μg kg^{-1}, $P = 0.055$ at 120 μg kg^{-1}). The increase in Peak Th was significantly greater post MC710 administration than after NovoSeven at 120 μg kg^{-1} (MC710 against NovoSeven:

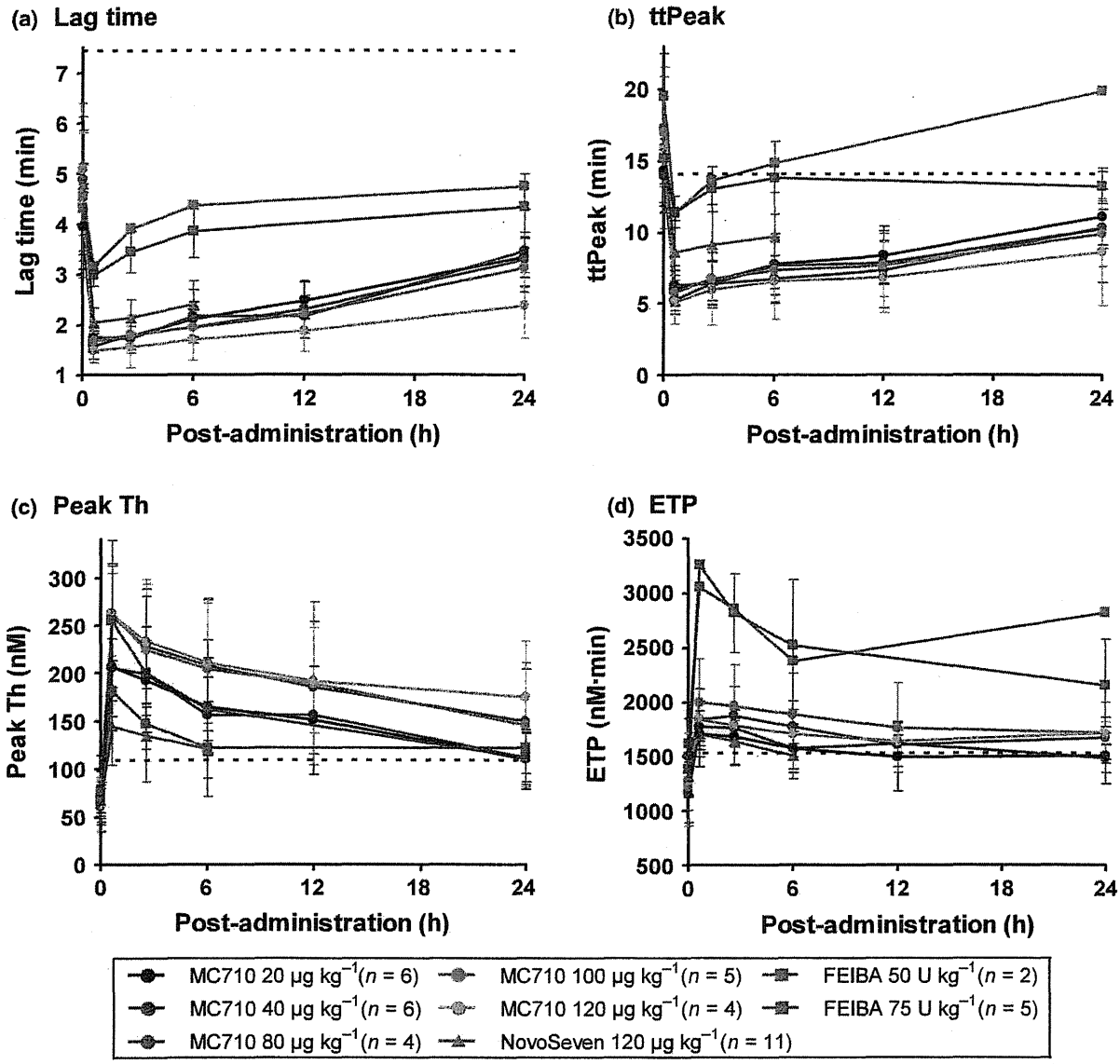

Fig. 5. Changes in TGT parameters. Time-dependent changes in TGT parameters obtained from thrombin generation profiles are shown. The assay was conducted using the plasma at pre and postadministration; 10 min, 2, 6, 12 and 24 h for five dose rates of MC710, 10 min, 2 and 6 h for NovoSeven, 10 min, 2, 6 and 24 h for FEIBA. (a) Lag time; (b) ttPeak; (c) Peak Th; (d) ETP (mean ± SD). The same symbols and lines are used in (a)–(d). Dashed lines in (a)–(d) indicate the mean values for NHP (18 times repetitive measurement). Parameter values for NHP (mean ± SD) are Lag time; 7.42 ± 0.27 min, ttPeak; 14.07 ± 0.43 min, Peak Th; 108.99 ± 8.92 nM and ETP; 1531.28 ± 73.27 nM min.

$P = 0.039$ at 20 μg kg^{-1}, $P = 0.041$ at 40 μg kg^{-1}, $P = 0.049$ at 80 μg kg^{-1}, $P = 0.031$ at 100 μg kg^{-1} and $P = 0.029$ at 120 μg kg^{-1}). Conversely, postadministration of FEIBA produced a marked increase in ETP; the effect was approximately double that of MC710 and NovoSeven. Enhanced ETP was significantly greater post FEIBA administration than after all doses of MC710 (75 U kg^{-1} FEIBA against MC710: $P = 0.002$ at 20 μg kg^{-1}, $P = 0.004$ at 40 μg kg^{-1}, $P = 0.005$ at 80 μg kg^{-1}, $P = 0.004$ at 100 μg kg^{-1}, $P = 0.003$ at 120 μg kg^{-1}).

Discussion

CWA with an MDA® II has been used to examine the pathophysiology of disseminated intravascular coagulation (DIC) [7,8], quantify trace concentrations of FVIII (<1.0 IU dL^{-1}) in the plasma of severe haemophilia A patients, and analyse the coagulation process induced by rFVIIa in clotting factor-deficient plasma [5,9]. In this study, we evaluated the effectiveness of CWA using plasma from haemophilia patients with inhibitors who were given bypassing products includ-

ing MC710. We confirmed the change in APTT post MC710 administration was dose dependent (Fig. 3a) and that the clot waveform profile at 10 min postadministration of MC710 at 120 μg kg^{-1} was close to that of NHP (Fig. 2a).

The values of |Min1| and |Min2| immediately postadministration of MC710 at doses of ≥ 80 μg kg^{-1} were greater than those for NovoSeven at 120 μg kg^{-1} g or FEIBA at 75 U kg^{-1} (Fig. 3b and c), suggesting that MC710 has superior bypassing activity. However, mean |Min1| and |Min2| were not MC710 dose-dependent and were higher at 100 μg kg^{-1} (given to five subjects) than at 120 μg kg^{-1} (given 4 subjects). The three of four subjects administered 120 μg kg^{-1} MC710 were not administered 100 μg kg^{-1}; therefore, the 100 μg kg^{-1} and 120 μg kg^{-1} subject groups were not considered the same, as this might influence the statistical analysis.

TGT evaluates thrombin production triggered by the TF/FVIIa complex. The coagulation reaction consists of (i) an initiation phase from the beginning of the coagulation reaction to the beginning of thrombin generation; (ii) a propagation phase with increasing thrombin production; and (iii) a termination phase, in which thrombin generation is suppressed by the protein C pathway or inhibitors. Lag time is an indicator of the initiation phase, and Peak thrombin and ttPeak reflect the propagation phase [10,11]. As shown in Figs 4a and 5a and b, Lag time and ttPeak were shortened in the NovoSeven and MC710 groups compared to NHP and FEIBA groups. TGT reflects extrinsic coagulation; thus, NovoSeven and MC710, which contain a large amount of FVIIa, were expected to have a shorter initiation phase. On the other hand, Peak Th was higher at all doses of MC710 than with NovoSeven at 120 μg kg^{-1} (Fig. 5c). These results suggest that explosive thrombin generation, which promotes the shift to the propagation phase, is more quickly established in plasma after administration of MC710 than with FVIIa alone. There was a more marked increase in ETP post FEIBA administration than after MC710 or NovoSeven, suggesting more sustained thrombin production is induced by FEIBA (Fig. 5d).

Clot waveform analysis evaluates the whole process of fibrin clot formation by thrombin generated in the 'intrinsic coagulation pathway' triggered by ellagic acid. TGT evaluates thrombin generation in the 'extrinsic coagulation pathway' using the TF/PL complex as a trigger of coagulation. Both methods directly or indirectly evaluate the amount of thrombin although the thrombin generation pathways differ. High doses of rFVIIa activate FX independently of TF in the absence of FVIII, generating a FXa-induced thrombin burst and thus providing bypassing activity [12–14]. FVIII or FIX in the intrinsic coagulation pathway does not function in APTT of the plasma of inhibitor patients; consequently, we conclude the FVIIa-activated reaction of FX depends on PL added to the measurement system. The specific characteristics of MC710 enable FVIIa to function more TF-independently by increasing the FX concentration in blood, resulting in changes in APTT. The increased FX concentration in the extrinsic coagulation pathway after MC710 administration also eased access of FX to the TF/FVIIa complex [15,16] and, as shown in the TGT results, the bypassing activity via TF by MC710 is expected to be greater than that achieved by monotherapy with FVIIa.

In this report, we described CWA and TGT in haemophilia patients with inhibitors after administration of bypassing agents NovoSeven, FEIBA and MC710. We concluded these measurement methods are appropriate for monitoring the procoagulant potential specific to bypassing agents and are available analytical tools for assessing bypassing activity in haemophilia patients with inhibitors. Measurements using both methods revealed MC710 at doses of ≥ 80 μg kg^{-1} induced higher |Min1| and |Min2| than did the control drugs; more rapid thrombin generation than FEIBA and greater thrombin production than NovoSeven. These results suggest that MC710 has superior bypassing activity in comparison to control drugs.

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Disclosures

Akira Shirahata and Yasuo Ohashi received a fee from KAKETSUKEN for the implementation of the trial. The other authors have no conflicts of interest to declare.

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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*.

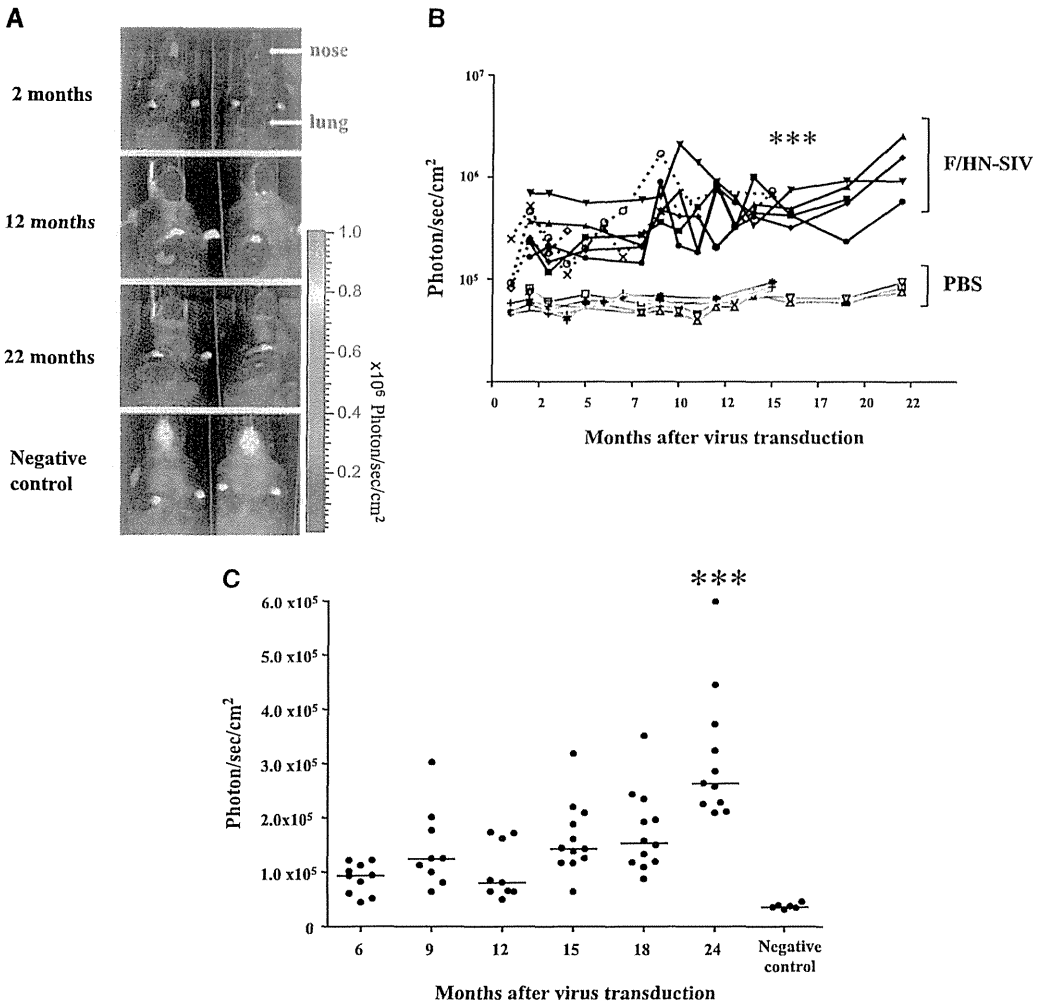

Figure 1. F/HN simian immunodeficiency virus (SIV) transduction leads to persistent gene expression in mouse lung. Mice were transduced with F/HN-SIV-luciferase (Lux) (5×10^8 TU/mouse) by nasal sniffing (n = 8 in two independent experiments) or received phosphate-buffered saline (PBS) (negative controls, 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