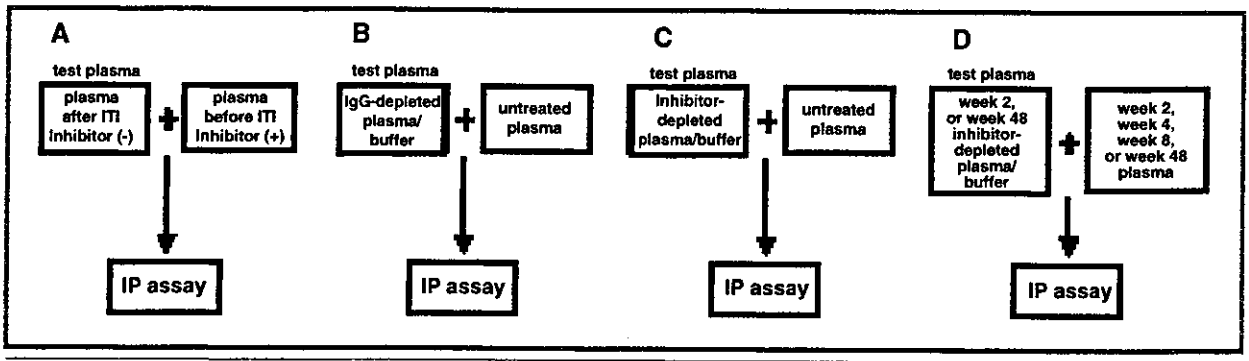

Figure 1 Detection of anti-FVIII antibody-neutralization activities.

IPU/mL were adjusted to give values of less than 10 IPU/mL.

Secondly, to confirm that the anti-FVIII antibody-neutralization factors in the patient's plasma were in immunoglobulins (IgGs), sample plasma pretreated with protein G-Sepharose beads (IgG-depleted plasma) or buffer A as control (30 mM $CaCl_2$, in 10 mM Tris-HCl, pH 7.2) was mixed with an equal volume of the same plasma without the protein G-Sepharose bead treatment (Figure 1B). The protein G-Sepharose beads for the elimination of IgGs were washed three times with one volume of buffer A containing 0.1% BSA, followed by 3 washes with one volume of buffer A without BSA. Fifty microliters of the patient's plasma were mixed with an equal volume of protein G-Sepharose beads (50% slurry) and incubated at 4°C with agitation overnight. Samples were centrifuged at 250×g for 3 min, and the supernatant was recovered as IgG-depleted sample plasma. The mixture of 50 µL of the patient's plasma and 25 µL of buffer A was incubated at 4°C with agitation overnight. This solution was used as a reference. The solutions were mixed with an equal volume of plasma from the same patient and incubated at 4°C with agitation for 3 hours. Anti-FVIII antibody titer was then measured by the IP assay.

Thirdly, to elucidate whether anti-FVIII antibody-neutralization antibodies existed in the patient's plasma, sample plasma pretreated with FVIII-Sepharose (i.e., anti-FVIII antibody-depleted plasma) or buffer A (control) was mixed with an equal volume of the same plasma without the FVIII-Sepharose treatment (Figure 1C). Sample plasmas were collected before ITI (patient D) and during ITI from the patients who received incompletely successful ITI (patients E, F, and G), and unsuccessful ITI (patients H and I). FVIII-Sepharose for elimination of anti-FVIII antibody was prepared. FVIII (3 mg) was coupled to 1 g CNBr-activated Sepharose 4B (Amersham Biosciences) in coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 8.3) at 4°C for 18 hours. Prior to use, the FVIII-Sepharose beads were washed three times with one volume of buffer A containing 0.1% BSA, followed by 3 washes with one volume of buffer A without BSA. Fifty µL of the patient's plasma were mixed with an equal volume of FVIII-Sepharose (50% slurry) and incubated at 4°C overnight with agitation. Samples were centrifuged at 250×g for 3 min, and the supernatant was recovered as anti-FVIII antibody-depleted sample plasma. The mixture of 50 µL of patient's plasma and 25 µL of buffer A was also incubated at 4°C with agitation overnight. This solution was used as a reference. The solutions were mixed with equal volume of the same patient's plasma and incubated at 4°C with agitation for 3 hours. Anti-FVIII antibody titer was measured by the IP assay.

Table 1 Inhibitor titer and outcomes of ITI in 9 inhibitor patients with hemophilia A.

	Weeks	*BU/mL*	*IPU/mL*	*Outcomes*
A	Before	2	52.5	
	After	0	0	
B	Before	1	85.6	Completely successful
	2	6	504	
	After	0	0	
C	Before	0	3.8	
	28	2	40.5	
	After	0	0	
D	Before	0	45.6	
	After	0	6.4	
E	Before	12	1644	
	3	234	12560	
	After	0	5.6	
F	2	44	3008	Incompletely successful
	4	23	684	
	8	2	148	
	After	0	3.6	
G	1	284	7890	
	2	756	19800	
	After	0	4.6	
H	1	1	7.6	
	2	123	940	
	After	30,4	528	Unsuccessful
I	1	930	30230	
	13	78	7340	
	After	2	48.8	

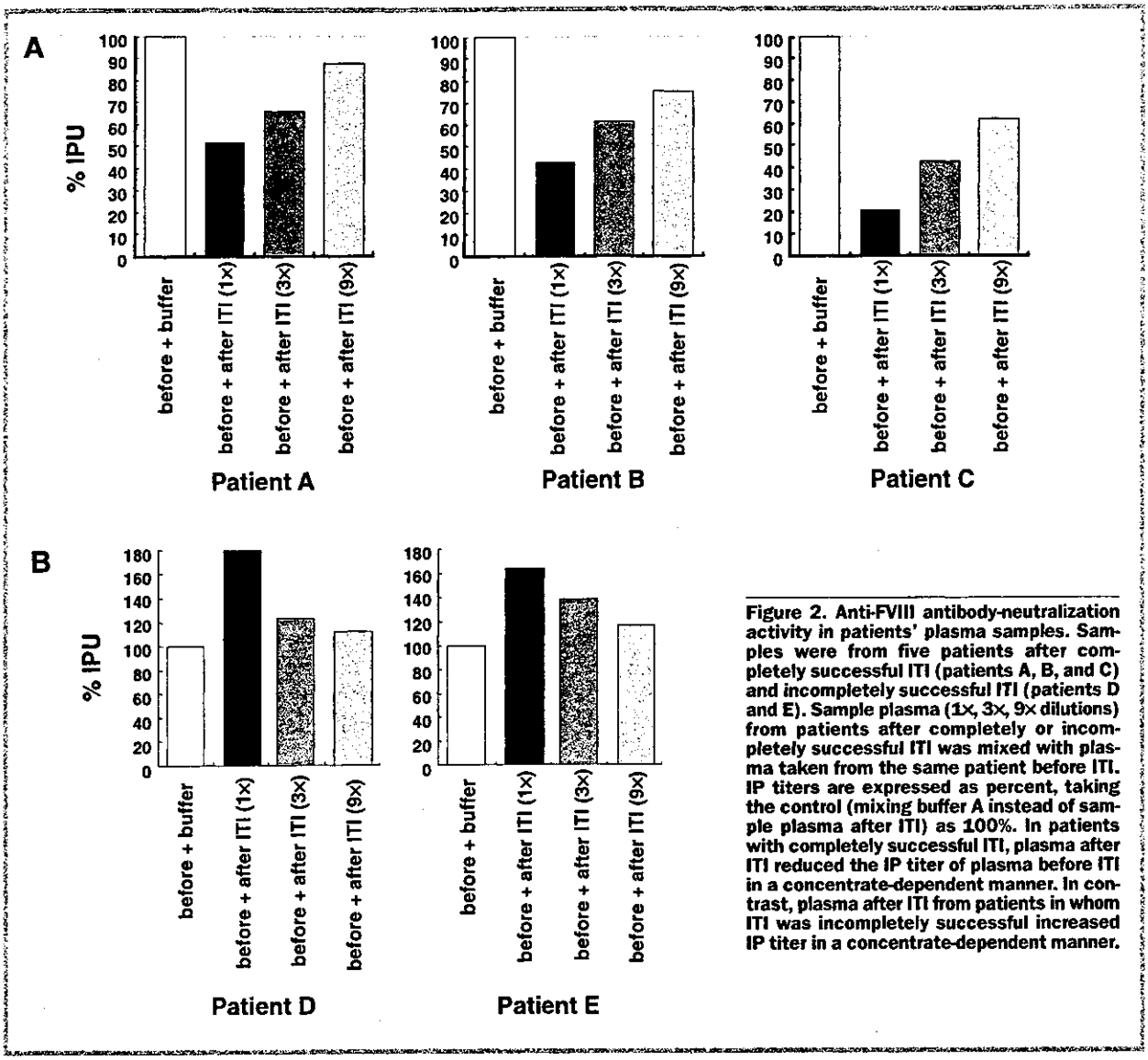

Figure 2. Anti-FVIII antibody-neutralization activity in patients' plasma samples. Samples were from five patients after completely successful ITI (patients A, B, and C) and incompletely successful ITI (patients D and E). Sample plasma (1×, 3×, 9× dilutions) from patients after completely or incompletely successful ITI was mixed with plasma taken from the same patient before ITI. IP titers are expressed as percent, taking the control (mixing buffer A instead of sample plasma after ITI) as 100%. In patients with completely successful ITI, plasma after ITI reduced the IP titer of plasma before ITI in a concentrate-dependent manner. In contrast, plasma after ITI from patients in whom ITI was incompletely successful increased IP titer in a concentrate-dependent manner.

Lastly, anti-FVIII antibody-depleted plasma obtained 2 and 48 weeks after the start of ITI or buffer A (control) was mixed with plasma obtained at four sampling points (week 2, 4, 8 and 48) during ITI from a patient who underwent incompletely successful ITI (Patient F) (Figure1D). Week 48 corresponds to the completion of ITI in this patient. After the mixture had been incubated at 4°C with agitation overnight, anti-FVIII antibody titer was measured by the IP assay.

Results

Inhibitory antibody and anti-FVIII antibody titer of patients

We investigated a total of 26 plasma samples from 9 hemophilic inhibitor patients who were treated with ITI (Table 1). Depending on BU and IP titers, patients were categorized into 3 groups, those in whom ITI was *completely successful,* whose IP and BU titers were undetectable in plasma after ITI, those in whom ITI was *incompletely successful* whose BU titer was undetectable but IP titer was still detected after ITI, and those in whom ITI was *unsuccessful* whose BU titer was detectable after ITI. The ITI was completely successful in three patients (A, B, and C), incompletely successful in four (D, E, F, and G) and unsuccessful in two (H and I).

Presence of anti-FVIII antibody-neutralization factor in the plasma from patients with completely successful ITI

Addition of plasma after ITI from patients with completely successful ITI to plasma taken from the same patients during ITI reduced IP titer in a dose-dependent manner (Figure 2A). On the other hand, the addition of plasma after ITI from two patients with incompletely successful ITI to plasma taken from the same patients during ITI produced a higher IP titer than that

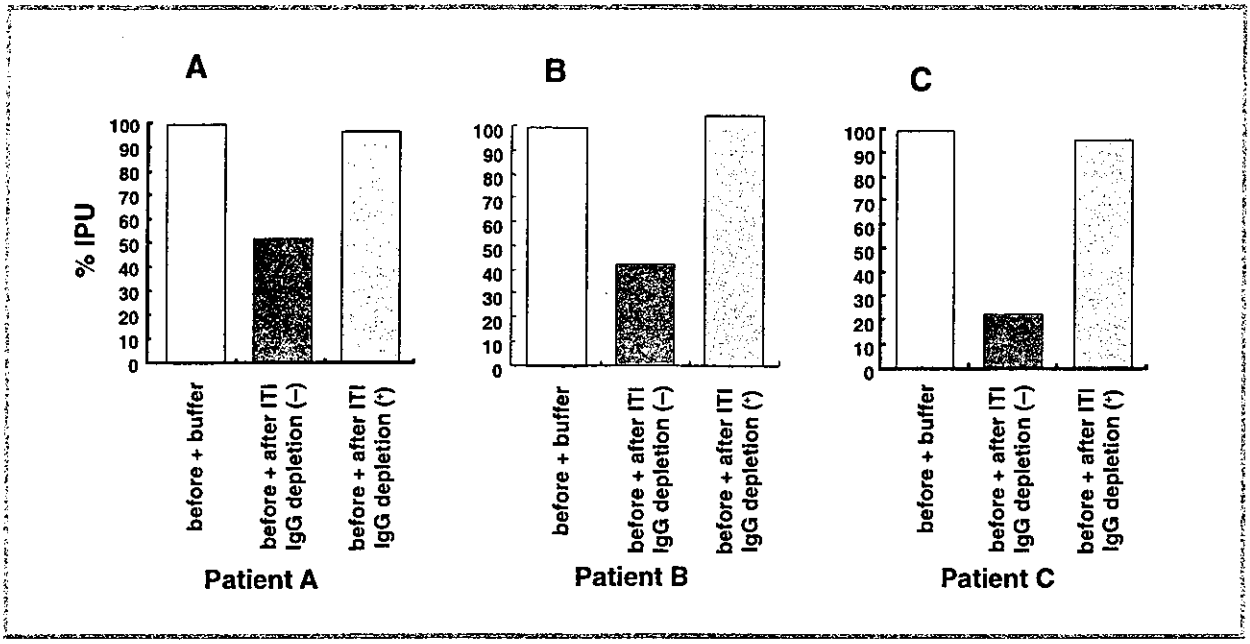

Figure 3. Effects of IgG depletion on inhibitor-neutralization activity. Samples were from three patients after completely successful ITI. IgGs were depleted from sample plasma using protein G-Sepharose beads. Sample plasma after ITI with/without IgG depletion was mixed with plasma taken from the same patient before ITI. IP titers are expressed as percent, taking the control (mixing buffer A instead of sample plasma after ITI) as 100%. In all cases, although sample plasma without IgG depletion after completely successful ITI did not affect IP titer, IgG depletion of sample plasma resulted in a reduction of the IP titer.

following addition of buffer A (control) (Figure 2B). This indicates that plasma from patients in whom ITI was completely successful contained some anti-FVIII antibody-neutralization factors.

Identification of anti-FVIII antibody-neutralization factor as IgGs

The effects of IgG-depleted plasma on IP titer of untreated plasma were investigated using plasma samples from the 3 patients in whom ITI had been completely successful. While plasma without protein G-Sepharose treatment reduced binding of antibodies to FVIII, IgG-depleted plasma did not influence it (Figure 3). Elimination of IgGs resulted in the loss of anti-FVIII antibody-neutralization effects, indicating that anti-FVIII antibody-neutralization factor would fall within IgGs. This implies that the plasma from patients with completely successful ITI contained IgGs against anti-FVIII antibodies, i.e. anti-idiotypic antibodies.

Anti-idiotypic antibodies existing in plasma during ITI

We also assessed the effects of anti-FVIII antibody-depleted plasma on IP titer of untreated plasma. All anti-FVIII antibody-depleted plasma samples reduced binding of antibodies to FVIII, while plasma without FVIII-Sepharose treatment did not influence, or even increased binding of antibodies to FVIII (Figure 4). These

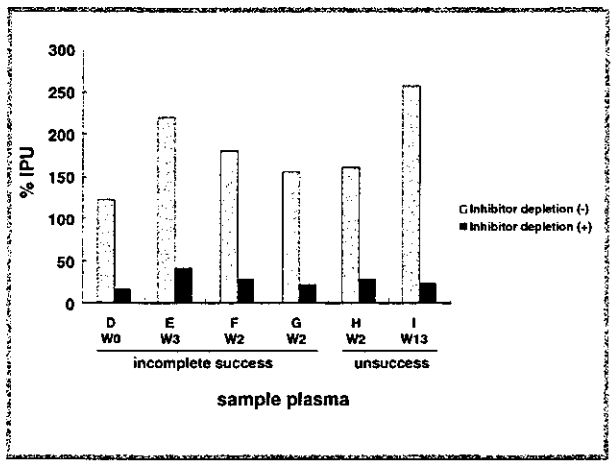

Figure 4. Effects of anti-FVIII antibody depletion on anti-FVIII antibody-neutralization activity. Samples were from four patients before (patient D, week 0) and during (patients E, week 3; patient F, week 2; and patient G, week 2) incompletely successful ITI, and from two during unsuccessful ITI (patients H, week 2; and patient I, week 13). Anti-FVIII antibodies were depleted from sample plasma using FVIII-Sepharose beads. Sample plasma before/during ITI with/without anti-FVIII antibody depletion was mixed with plasma obtained at the corresponding time point. IP titers are expressed as a percent, taking the control (mixing buffer A instead of sample plasma) as 100%. In all cases, although sample plasma without FVIII-Sepharose treatment increased IP titer, anti-FVIII antibody depletion from sample plasma resulted in a reduction of the IP titer.

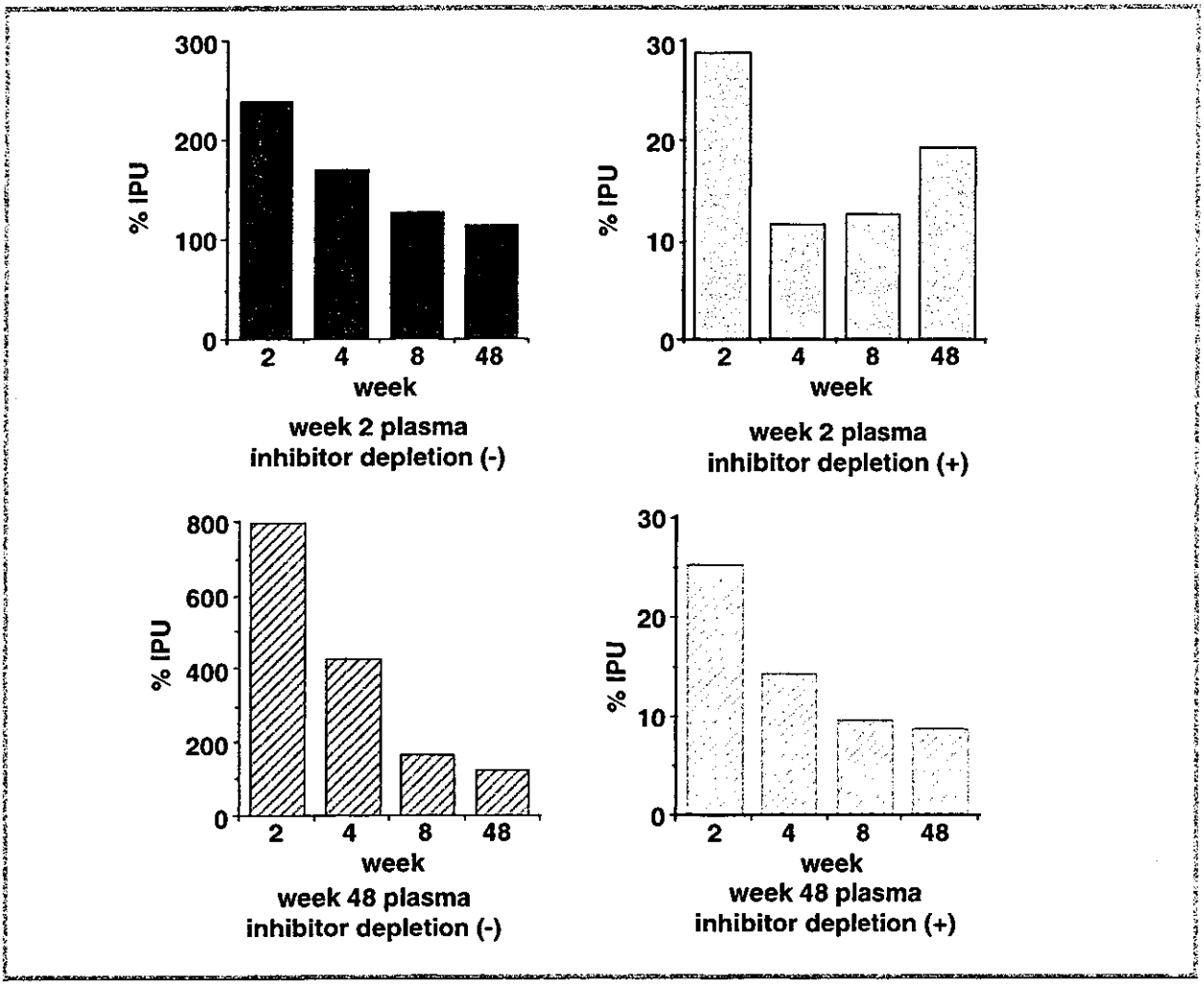

Figure 5. Alteration of anti-FVIII antibody-neutralization activity with the course of ITI. Samples were from a patient in whom ITI was incompletely successful. Anti-FVIII antibodies were depleted from sample plasma using FVIII-Sepharose beads. Sample plasma obtained at weeks 2 and 48 with/without anti-FVIII antibody depletion was mixed with plasma obtained at weeks 2, 4, 8 and 48. IP titers are expressed as a percent, taking the control (mixing buffer A instead of sample plasma) as 100%. Although sample plasma without anti-FVIII antibody depletion increased IP titer in both cases of adding week 2 and week 48 plasmas, anti-FVIII antibody depletion from sample plasma resulted in a reduction of the IP titer. Anti-FVIII antibody-depleted week 2 plasma decreased IP titer in plasma samples in a time-independent manner, while anti-FVIII antibody-depleted week 48 plasma decreased IP titer in a time sequence.

results imply that anti-idiotypic antibodies existed in plasma before and during ITI from patients in whom ITI was incompletely successful or unsuccessful.

Alterations of anti-idiotypic antibodies during ITI

Addition of anti-FVIII antibody-depleted plasma reduced IP titer in all cases (Figure 5), whereas addition of week 2 or week 48 untreated plasma increased IP titer compared to that of each control to which buffer A had been added. Anti-FVIII antibody-depleted week 2 plasma maximally reduced IP titer of week 4 plasma. Meanwhile, anti-FVIII antibody-depleted week 48 plasma reduced IP titer of week 2, 4, 8, and 48 plasma samples, in a time sequence.

Discussion

Idiotype refers to the assembly of antigenic determinants which are located in the variable regions of antibodies or antigen-specific receptors of T cells. Each idiotype contains the determinants that are specific to the antibody molecule or T-cell receptor, which is called *idiotope*. Corresponding anti-idiotypic antibodies can develop against an immunogenic idiotope. Idiotypes and anti-idiotypic antibodies are natural components of immune responses, and exert a regulatory role which maintains the homeostasis of the immune system. These interactions between idiotypes and anti-idiotypic antibodies constitute an idiotypic network. Immunological tolerance might be found in the subtle equilibrium

between anti-FVIII and corresponding anti-idiotypic antibodies. Since normal individuals produce both anti-FVIII and corresponding anti-idiotypic antibodies,[25,26] the cause of the tolerance to FVIII is not limited to deletion of self-reactive B and T cells.[27] It might thus be fruitful to investigate whether an idiotypic network plays a role in establishing and maintaining tolerance.

The immunoprecipitation method used in the present study, originally used to measure levels of all anti-FVIII antibodies in plasma samples, is highly sensitive and is independent of inhibitor activity measurable by the Bethesda method. By testing plasma samples from patients in clinical trials, 5% to 10% of patients without a detectable inhibitor by the Bethesda method have a significant immune response which ranges from 3-fold to 100-fold above the background.[28,29] It has been reported that the half-life of transfused FVIII is reduced as the anti-FVIII antibody concentration detected by an ELISA, which is as sensitive as the IP assay, increases in non-inhibitor patients with hemophilia A.[30] This study has a limitation, but indicates that some of the inhibitor-negative patients may have symptom exacerbation as a result of a rise of anti-FVIII antibodies.

In the present study, in order to elucidate whether plasma from patients after ITI contained anti-idiotypic antibodies, simple mixing studies were performed using the immunoprecipitation method. In the previous studies the detection method generally used was a solid-phase ELISA. We developed a novel liquid phase blocking IP method, which should provide more reliable results. Anti-FVIII antibody-neutralization activity was observed in plasma from patients in whom ITI was completely successful, but not in plasma from those in whom ITI was incompletely successful or unsuccessful, suggesting the presence of anti-FVIII antibody-neutralization factor in plasma from the completely successfully ITI treated patients. The second assays revealed that IgG-depleted plasma from patients with completely successful ITI contained no anti-FVIII antibody-neutralization factor. Furthermore, the third assays proved the presence of anti-idiotypic antibodies even in plasma samples that showed positive inhibitor titer. These results suggest that anti-FVIII antibody-neutralization factor is an IgG; i.e., the anti-FVIII antibody-neutralization factor would be anti-idiotypic antibodies against anti-FVIII antibodies, and anti-idiotypic antibodies exist at any time in any hemophilic patients who receive FVIII replacement therapy.

The last mixing studies using one patient's plasma samples taken at different time points provided the possibility to examine the affinity maturation of IgG anti-idiotypic antibodies. Anti-FVIII antibody-depleted plasma obtained during an early period of ITI (week 2 plasma) reduced IP titer in plasma samples in a time independent manner. On the other hand, the anti-FVIII antibody-neutralization activity of anti-FVIII antibody-free plasma obtained at completion of ITI increased over time. These results indicate that neutralization activity of anti-idiotypic antibody increases over the course of ITI. Affinity maturation of IgG antibodies in adaptive immune responses is a well-accepted mechanism to improve effector functions of IgG within 2 weeks to several months after antigen encounter. Idiotypic suppression function is a driving force for diversification and maturation of the antigen-induced response.[31] Further studies will be necessary to clarify the role of affinity maturation of anti-idiotypic antibodies in ITI.

Our observations suggest that, in patients whose IP titer was suppressed by ITI, anti-idiotypic antibodies shifted the immune system toward the steady-state equilibrium that prevented alloimmunity in hemophilia A patients with inhibitors. The present study thereby contributes to a better understanding of the association of anti-idiotypic antibodies with ITI.

YS was responsible for the conception and design of the study, for acquisition, analysis and interpretation of data and for drafting the article. IT, KF and KY were responsible for acquisition, analysis and interpretation of data. MS and AY were responsible for conception of the study, enrolling patients and interpretation of data. All authors drafted or critically revised the manuscript, and all authors approved the final version of the manuscript. The authors reported no potential conflict of interest.

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Effectiveness of factor VIII infusions in haemophilia A patients with high responding inhibitors

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Summary. We report here the efficacy of factor VIII (FVIII) infusions in two haemophiliacs with inhibitors using clot waveform analysis on the MDA® II system, which was possible to detect very low levels of FVIII activity < 1.0 U dL^{-1}. In the presence of type 1 inhibitors at the level of 6.2 (patient 1) and 14.4 (patient 2) Bethesda Units mL^{-1}, 3.2 and 6.5 U dL^{-1} of FVIII:C remained 30 min after the infusion of FVIII (100 U kg^{-1}), respectively. Moreover, 0.9 U dL^{-1} of FVIII:C remained 24 h after infusion in patient 2. In both cases, these changes were reflected by qualitative improvement in the aPTT clot waveform and quantitative changes in the minimum value of the second derivative of the aPTT waveform (Min2) that reflects clot acceleration. These results suggest that FVIII infusion may be continued with clinical benefit in some haemophiliacs with high responding inhibitors. Furthermore, the haemostatic response may be monitored accurately and efficiently by clot waveform analysis.

Keywords: clot waveform analysis, haemophilia A, high responders, immune tolerance induction, inhibitor

Introduction

Alloantibodies to factor VIII:C (FVIII:C) develop in approximately 20–30% of patients with severe haemophilia A who have been treated with FVIII replacement therapy [1,2]. These patients can be divided into two groups: high responders, demonstrating an anamnestic inhibitor response after exposure to FVIII and low responders who show no significant inhibitor response after challenge with factor VIII [3]. Replacement therapy with FVIII concentrates in patients with inhibitors often becomes ineffective, thus complicating clinical management. Therefore, they are usually treated with (activated) prothrombin complex concentrates [(A)PCCs] or recombinant factor VIIa, i.e. FVIII bypassing therapy [4,5]. Alternatively, immune tolerance induction (ITI) may be attempted to reduce or eradicate the inhibitor activity [6–8]. Several investigators have reported that tolerance may be achieved in approximately 76–89% of patients with inhibitors [6–8]. The reduction in inhibitor level was associated with a decrease in the number of bleeding episodes and an improved quality of life. Unfortunately, some high responders do not respond sufficiently to ITI. Inhibitor levels in such patients often fluctuate for long periods of time. As the dose of FVIII infused regularly does not appear to neutralize the inhibitor sufficiently, it might be presumed that it would also be haemostatically ineffective in these patients. Nevertheless, clinical evaluation frequently suggests a subjective improvement in the severity of the disease even when ITI is not completely successful. This suggests the possibility that the regular infusion of FVIII might decrease the number of bleeding episodes in high responders. Clearly it would be useful to document this subjective clinical assessment of improvement with objective measurements of the changes in plasma levels of FVIII:C achieved following FVIII infusion. Unfortunately, monitoring of the very low levels of FVIII:C achieved in such circumstances has been limited by the sensitivity of the FVIII:C assay systems available. Recently, however, we have demonstrated that using the MDA® II Hemostasis system (bioMérieux, Durham, NC,

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USA), a photo-optical automatic coagulation analyzer, very low levels of FVIII can be measured relatively accurately [9]. Moreover, due to the systems capability of performing clot waveform analysis, it is useful for the evaluation of dynamic changes in blood coagulation [10,11]. Consequently, qualitative as well as quantitative comparisons may be made both between patients and within a patient during the response to therapeutic intervention [9]. In this study, we examined whether this approach may be used to document objectively the clinical efficacy of FVIII infusion in two high responder patients, who appeared to respond insufficiently to ITI treatment as assessed by their inhibitor response.

Materials and methods

Patients

Patient 1 was diagnosed as having severe haemophilia A when he experienced recurrent subcutaneous haematomas at 4 months of age. An FVIII:C inhibitor (1.2 BU mL^{-1}) was detected for the first time at 13 months. The maximum inhibitor level recorded before ITI was attempted was 5.0 BU mL^{-1} and was 2.0 BU mL^{-1} immediately prior to its initiation. At the age of 9, ITI was attempted with the intermediate dose regimen of our facility: a daily administration of 4000 U (100 U kg^{-1}) of recombinant FVIII (Recombinate™; Baxter Healthcare Corporation, Westlake, CA, USA) for 3 weeks, followed by an infusion four times a week. The maximum inhibitor level after ITI was 152.0 BU mL^{-1}. The inhibitor level had fluctuated for 3 years since ITI was initiated. Although the increases in the inhibitor titre were seen at 14 and 36 months without any clinical events such as infection, the regular infusion of FVIII was continued. Infusion studies were performed 5, 19 and 33 months after the initiation of ITI (Fig. 1a).

Patient 2 was diagnosed as having severe haemophilia A when he experienced recurrent subcutaneous haematomas and purpura 4 months after birth. A Factor VIII:C inhibitor (3.2 BU mL^{-1}) was detected for the first time at 23 months. The maximum inhibitor level recorded before ITI was attempted was 62.0 BU mL^{-1} and was 3.9 BU mL^{-1} immediately prior to its initiation. At the age of 9, ITI was attempted with a daily administration of 4000 U (100 U kg^{-1}) of recombinant FVIII (Recombinate™) for 3 weeks, followed by infusion three times a week. The maximum inhibitor level after ITI was 17.4 BU mL^{-1}. The inhibitor activity had not fallen below 4 BU mL^{-1} for 2 years since ITI was initiated.

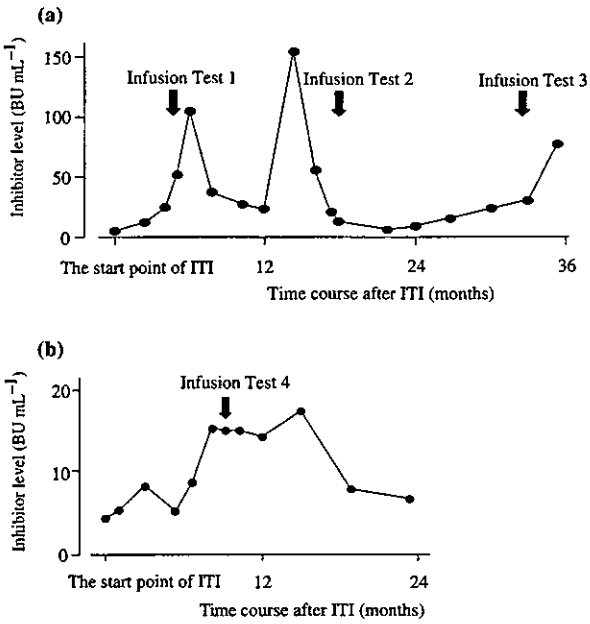

Fig. 1. Inhibitor levels after the initiation of ITI. (a) Patient 1, (b) patient 2. Closed arrows represent the dates of each infusion test. BU, Bethesda Unit; ITI, immune tolerance induction.

An infusion study was performed 9 months after the initiation of ITI (Fig. 1b).

Informed consent for the study was obtained from the patients and their parents.

Blood sample preparation

Nine volumes of whole blood were withdrawn into 1 volume of 3.8% (w/v) trisodium citrate solution. Platelet-poor plasma was separated by centrifugation at 2500 *g* for 15 min. The plasma obtained was frozen immediately in ethanol solution cooled by dry ice and stored at −70 °C until the time of study.

FVIII:C inhibitor assay and characterization

Inhibitor assays were performed using the Bethesda method as described by Kasper *et al.* [12]. The mode of FVIII:C inactivation was determined by reaction kinetics between FVIII:C and the inhibitors as described by Biggs *et al.* [13,14]. The FVIII-specific IgG subclass of the alloantibodies was determined by enzyme-linked immunosorbent assay as previously described [15]. Immunoblot analysis was performed as previously described [16].

Activated partial thromboplastin time (aPTT) test

The aPTT was performed on the bioMérieux MDA® II Hemostasis System using MDA® reagents (Platelin®

LS, Platelin® LS $CaCl_2$) supplied by the manufacturer (bioMérieux). Verify® 1 (bioMérieux) calibrated against the international standard was used as the normal control plasma.

FVIII:C assay

FVIII:C assay was performed by the one-stage clotting method on the MDA® II system using FVIII:C-deficient plasma (bioMérieux) as the substrate [17]. The standard curve was prepared using the Verify® 1 reagent in serial doubling dilutions from 1:10 to 1:5120. The patient (test) plasmas were diluted to 1:10. One hundred units (U) of FVIII:C was defined as the amount of FVIII:C activity present in 1 dL of normal plasma (Verify® 1). The detection limit of this assay system was 0.2 U dL^{-1} [9].

aPTT waveform analysis

Clot waveform analysis of the optical data from the aPTT test was performed as described by Braun *et al.* [11]. The waveform recorded was automatically processed mathematically by the MDA® II system, using algorithms built into the software. The data obtained were accessed using waveform information and export research tools (WIT®/WET®) provided by bioMérieux.

The aPTT and the minimum value of the second derivative (Min2) of the aPTT waveform, i.e. the percentage change in light transmission (T) over time (S^2) reflecting the acceleration in fibrinogen to fibrin conversion at the point in time that clotting is initiated, was measured in all cases.

Infusion studies

The studies were performed in both patients before and following one of their scheduled FVIII infusions during their ITI maintenance protocol, i.e. 100 U kg^{-1} of recombinant FVIII (Recombinate™) four times weekly in patient 1 and three times weekly in patient 2. Blood samples for the determination of aPTT, Min2 and FVIII:C assay were taken from the patients at each time point before and after the bolus infusion of 100 U kg^{-1} of FVIII; three time points (pre-, 30 min and 24 h postinfusion) for infusion test 1and 2 in patient 1, and infusion test 4 in patient 2, whereas six time points (pre-, 15 min, 30 min, 1 h, 6 h and 24 h postinfusion) for infusion test 3 in patient 1. Neither patient had received FVIII concentrates or (A)PCC for 48 h before the initiation of each infusion test.

Results

Characterization of inhibitors

The antibodies in both patients were considered to be type 1 inhibitors with complete inactivation of FVIII:C characterized by the reaction kinetics between FVIII:C and the inhibitors. The main IgG subclass of patient 1's inhibitor was IgG_4 with some IgG_1 and IgG_2 subclasses, whereas in patient 2 the antibodies were predominantly of the IgG_4 subclass. Immunoblot analysis revealed that in patient 1 the antibodies recognized both the heavy (44 kDa fragment) and light chains (72 kDa fragment) of FVIII, whereas in patient 2 the antibodies recognized only the heavy chain (44 kDa fragment). Thus, both sets of inhibitors showed the typical patterns of FVIII alloantibodies.

Infusion studies

In patient 1, separate infusion studies were performed 5, 19 and 33 months after ITI was initiated when the inhibitor levels were 50.0, 6.2 and 27.0 BU mL^{-1}, respectively (Fig. 1a). In the first study (infusion test 1), there was no improvement in FVIII:C level, Min2 and the aPTT waveform after the infusion of FVIII (Table 1, Fig. 2b). In the second study (infusion test 2), when the inhibitor level was 6.2 BU mL^{-1}, 3.2 U dL^{-1} of FVIII:C was detected

Table 1. Coagulation tests before and after FVIII infusion.

	Patient 1						Patient 2			
	Infusion test 1			Infusion test 2			Infusion test 4			
	Before	30 min after	24 h after	Before	30 min after	24 h after	Before	30 min after	24 h after	Normal control (mean ± SD)
aPTT (s)	119.9	119.9	121.0	109.3	68.7	105.8	132.3	52.2	64.8	29.2 ± 1.2
Min2	10.0	10.0	9.8	11.0	56.0	14.0	9.0	93.0	62.0	218 ± 21
FVIII:C (U dL^{-1})	<0.2	<0.2	<0.2	<0.2	3.2	<0.2	<0.2	6.5	0.9	
Inhibitor titre (BU mL^{-1})	50.0			6.2			14.4			

aPTT, activated partial thromboplastin time; BU, Bethesda unit.

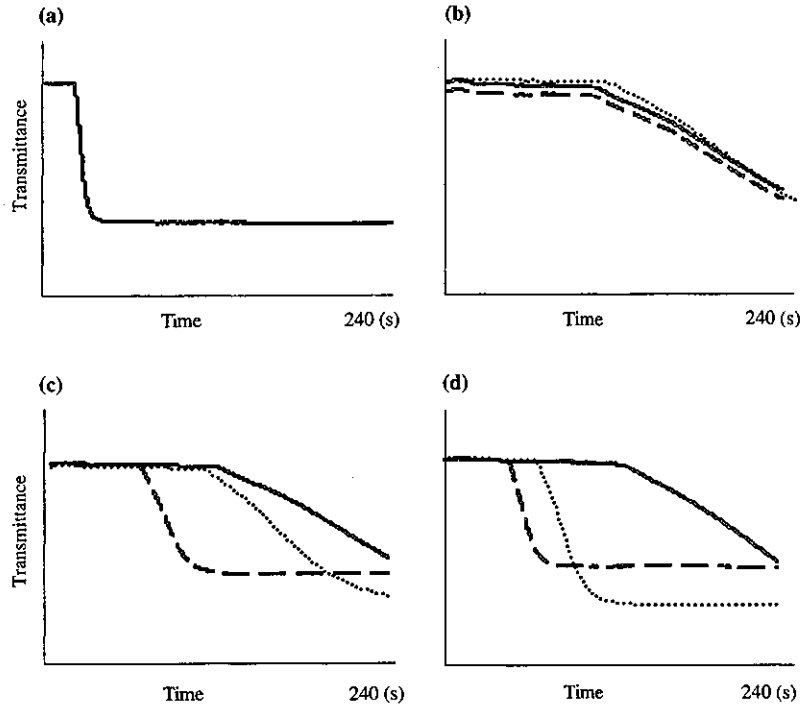

Fig. 2. aPTT clot waveforms observed in patients 1 and 2 pre- and post-FVIII infusion (a) Normal control, (b) patient 1 (infusion test 1); (c) patient 1 (infusion test 2); (d) patient 2 (infusion test 4). The MDA® II Hemostasis system plots and exports a hard copy of a continuous tracing of the changes in light transmission over time that occur during the performance of the aPTT. The aPTT waveforms obtained from a single test before (solid line), 30 min after (broken line) and 24 h after (dotted line) the infusion of 100 U kg^{-1} of FVIII into each patient are shown.

Table 2. Coagulation tests in the time course study in patient 1 after FVIII infusion.

	Infusion test 3 in patient 1						Normal control
	Before	15 min after	30 min after	1 h after	6 h after	24 h after	(mean ± SD)
aPTT (s)	120.4	67.2	68.0	69.6	77.1	112.7	29.2 ± 1.2
Min2	11.5	62.5	60.0	57.5	49.0	19.5	218 ± 21
FVIII:C (U dL^{-1})	< 0.2	2.4	2.3	1.9	1.0	< 0.2	
Inhibitor titre (BU mL^{-1})	27.0						

aPTT, activated partial thromboplastin time; BU, Bethesda unit.

30 min postinfusion with the significant improvement in Min2 and the aPTT waveform (Table 1, Fig. 2c). In the time course study (infusion test 3), when the inhibitor level was 27.0 BU mL^{-1}, the detection of FVIII:C and the improvement of Min2 were observed for at least 6 h postinfusion (Table 2).

In patient 2, only one infusion study (infusion test 4) was performed 9 months after ITI was initiated when the inhibitor level was 14.4 BU mL^{-1} (Fig. 1b). The FVIII:C levels 30 min and 24 h postinfusion were measured to be 6.5 and 0.9 U dL^{-1}, respectively. The improvement of Min2 and the aPTT waveform were observed 30 min after, even 24 h after the infusion of FVIII (Table 1, Fig. 2d).

Clinical evaluation

After the initiation of regular infusions of FVIII for ITI treatment, the number of bleeding episodes decreased in patient 1 from 26–35 to five to eight episodes over a 1-year period. Similarly, in patient 2, the number of bleeding episodes observed over a year decreased from 13–18 to six to seven episodes per year. The increased inhibitor level at 14 and 36 months in patient 1 seemed to have no adverse effect on bleed frequency.

Discussion

When no significant fall in the inhibitor level is observed in high responders 2–3 years after the initiation of ITI, it is generally considered necessary to abandon conventional FVIII concentrate replacement therapy in favour of expectant FVIII bypassing therapy for bleeding episodes as and when they occur [3]. Nonetheless, some investigators have advocated continuing with conventional replacement therapy albeit at significantly increased dosage to achieve the

desired haemostatic effect [18]. Studies of these two patients confirmed our previous experience that indeed the clinical status of such patients, as assessed by the incidence of bleeding episodes, might be improved by the regular infusion of FVIII despite the persistence of relatively high levels of alloantibodies to FVIII:C. However, on this occasion we were also able to document that the improvement, measured by relatively subjective clinical assessment, was associated with objective improvement in their coagulation status measured *ex vivo*. This was assessed qualitatively using clot waveform analysis and quantitatively using the aPTT, Min2 and one-stage FVIII:C assay on the MDA® II Hemostasis system.

In patient 1, when his inhibitor level was 50.0 BU mL^{-1} and probably rising (Fig. 1a), infusion of 4000 U (100 U kg^{-1}) of recombinant factor VIII was not associated with change in any of the measured parameters 30 min after the infusion. However, 14 months later when the inhibitor level was substantially lower (6.2 BU mL^{-1}) (Fig. 1a), infusion of the same dose of FVIII was associated with normalization of his clot waveform profile (Fig. 2), substantial but incomplete correction of the aPTT and Min2 and a measurable level of FVIII:C in the one-stage assay. In the time course study, when the inhibitor level was 27.0 BU mL^{-1} (Fig. 1a), the improvement of the aPTT and Min2 and the detection of FVIII:C had been observed for up to 6 h postinfusion. These observations are of particular importance as the level observed, moved him from the severe to moderate category of severity of haemophilia A at least 6 h following the infusion of FVIII. Theoretically, this would be expected to be associated with a reduction in the bleeding episodes experienced, which was in fact observed. These observations in patient 1 during the second and third infusion tests were generally mirrored by the observations in patient 2. Moreover, in this patient one-stage FVIII:C assay detected a concentration of FVIII (0.9 U dL^{-1}) 24 h postinfusion. Very low levels of FVIII:C < 1.0 U dL^{-1} might remain to be neutralized completely in the presence of some type 1 inhibitors. Using the MDA® II assay system allowed the evaluation of very low levels of FVIII:C < 1.0 U dL^{-1} [9]. Again, this level is at the cut-off between the severe and moderate categories of severity and is in line with the improved clinical status of this patient.

[1]The neutralized amount of FVIII was calculated by the following formula: body weight (kg) × 20 × inhibitor titre (BU mL^{-1})

On theoretical grounds, the amount of FVIII that could be neutralized in patients 1 (infusion tests 2 and 3) and patient 2 (infusion test 4) would be approximately 5000, 21 600 and 11 500 U, respectively.[1] In these cases, we have continued regular infusion of 4000 U FVIII not for neutralization of the inhibitors, but for ITI treatment, resulting in the decrease in bleeding episodes by the prophylactic effect. Analysis of the inhibitor epitopes revealed that the antibodies in patient 1 recognized both A2 and C2 domains of FVIII, whereas the antibodies in patient 2 recognized A2 domain. Both A2 and C2 domains are common binding regions for FVIII alloantibodies [3]. The difference in response observed in patient 1 following the three infusion tests may be important. When the inhibitor level was 50.0 BU mL^{-1}, the infusion was ineffective and the coagulation parameters were unchanged, whereas 3.2 and 2.3 U dL^{-1} of FVIII:C was detected 30 min after the infusion of FVIII in the presence of 6.2 and 27.0 BU mL^{-1} of inhibitor, respectively. These data suggest that the response may depend on the inhibitor level. This in turn may reflect the development of anti-idiotypic or non-neutralizing antibodies during ITI treatment, which may be interfering with the binding of the inhibitors with FVIII infused. Clearly, more detailed studies are needed to confirm this hypothesis.

In conclusion, our studies confirm that the regular infusion of FVIII might still be a therapeutic option in high responders, whose inhibitor level is < 30 BU mL^{-1}. They also demonstrate further evidence that clot waveform analysis provides an important tool in measuring objectively *ex vivo* changes in coagulation status that are otherwise inaccessible [9]. In patients such as those described here it is clearly important to correlate perceived clinical improvement with objective tests of haemostatic function wherever possible. The tests described are simple to perform and provide relatively immediate guidance to the prescribing doctor as to the feasibility of continuing with conventional therapy. Further and more controlled clinical study of this approach would appear to be justified.

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Successful Gene Transfer Using Adeno-Associated Virus Vectors into the Kidney: Comparison among Adeno-Associated Virus Serotype 1–5 Vectors in vitro and in vivo

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Key Words
β-Galactosidase · Dependovirus · Epithelial cell · Gene therapy, adeno-associated virus serotype 1–5 vectors · Viral vectors, gene therapy

Abstract
Background/Aim: Gene transfer into the kidney has great potential as a novel therapeutic approach. However, an efficient method of gene transfer into the kidney has not been established. We explored the transduction efficiency of renal cells in vitro and in vivo using adeno-associated virus (AAV) serotype 1–5 vectors encoding the β-galactosidase gene. ***Methods:*** In the in vitro study, rat kidney epithelial cell line NRK52E cells were transfected with AAV serotype derived vectors. In the in vivo study, AAV serotype derived vectors were selectively injected into the kidney using a catheter-based gene delivery system in rats and mice mimicking the clinical procedure. The efficiency of gene expression was histologically evaluated on the basis of the β-galactosidase expression. ***Results:*** AAV serotype 1, 2, and 5 vectors transduced in rat kidney epithelial cell line NRK52E cells in vitro, whereas AAV serotype 3 or 4 vectors showed no transduction. In addition, the kidney-specific injection of AAV serotype 2 vectors successfully transduced in tubular epithelial cells, but not in glomerular, blood vessel, or interstitial cells in vivo, whereas the rest of the serotypes showed no transduction. ***Conclusion:*** Since kidney-specific gene delivery via the renal artery by catheterization is highly feasible in humans, these findings provide useful information for promising strategies in renal gene therapy.

Introduction

Gene transfer into the kidney has a great potential as a novel therapeutic approach for renal diseases. However, the ability to pursue gene therapy for renal diseases is substantially limited, because there is no efficient and adequate local gene delivery system into the kidney and because of the potential adverse effects on renal functions

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associated with systemic gene therapy. A few reports have been published on gene delivery into the kidney using recombinant virus vectors in experimental models [1, 2]. The direct injection of retrovirus vectors into the kidney has been reported to transduce in tubular epithelial cells, but not in glomerular or vascular cells [3]. Because retrovirus vector mediated gene transduction requires target cell replication, the use of retrovirus vectors in renal gene therapy is still restricted. The adenovirus vector is an alternative vector system that results in high gene transduction in both dividing and nondividing cells. Several investigations demonstrated that adenovirus vectors were successfully transduced in renal cells [4–6]. However, the clinical application of adenovirus vectors also has been limited by cytotoxicity, immune responses, and short-term transgene expression.

Adeno-associated virus (AAV) vectors have a number of attractive features for the clinical application of renal gene therapy: no cytotoxicity, the ability to transduce in both dividing and nondividing cells, low immune responses, and long-term transgene expression [7–9]. When the experiment was started, there were six primary isolates of AAV (AAV serotypes 1–6) [8, 10–14], although the cap sequence of serotype 6 is strongly related to that of serotype 1 [8]. Two novel serotypes were recently isolated from monkeys [15]. The distinct AAV serotypes were shown to have different tropisms for the cells and tissue. For instance, the AAV serotype 1 vectors could efficiently transduce the skeletal muscles [16], whereas AAV serotype 5 vectors could efficiently transduce liver [17] and central nervous system [18]. Although these differences in the tropism might be due to the receptors and processes during AAV transduction, the precise mechanisms are unknown. In terms of AAV vector mediated gene transfer into the kidney, it is not known whether any of the serotypes of AAV vectors can achieve transduction. In the present study, we explored the transduction efficiency of renal cells in vitro using AAV serotype 1–5 vectors. To achieve efficient gene transduction in vivo, we developed a catheter-based injection system that can be inserted into the kidney of rats and mice and tested these distinct AAV serotype vectors.

Materials and Methods

Cell Culture and Reagents

Normal rat kidney cell lines (NRK52E and ACTT-CRL1571) displaying a tubuloepithelial phenotype [19] were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were plated onto 60-mm dishes (Falcon, Franklin Lakes, N.J., USA) at a density of 3×10^6 and cultured at 37°C in 5% carbon dioxide and 95% air. The primary antibody against aquaporin-1 (AQP-1) was prepared with rabbit immunization, targeting for rat AQP-1 (234–247; Arg-Ser-Ser-Asp-Phe-Thr-Asp-Arg-Met-Lys-Val-Trp-Thr-Ser). The reagents were obtained from Sigma (St. Louis, Mo., USA) unless otherwise indicated.

Experimental Animals

Twelve male Lewis rats (initial body weight 110–150 g), originally purchased from Japan SLC (Shizuoka, Japan) and maintained at our animal center, and 10 male BALB/c mice (initial body weight 27–33 g), purchased from CLER Japan (Tokyo, Japan), were used in this study. The animals had free access to standard chow and drinking. All experiments in this study were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Preparation for AAV Vectors

AAV vectors encoding the β-galactosidase (β-gal) gene were produced based on plasmid transfection [10]. Briefly, subconfluent 293 cells were cotransfected with AAV vector plasmid, AAV helper plasmid, and adenovirus helper plasmid by a calcium phosphate precipitation method. Recombinant AAV was harvested by three cycles of freezing/thawing. The vector solution was then purified twice on a gradient. The vector titer was determined by quantitative dot-blot hybridization of DNase-treated stocks.

Detection of β-Gal Expression

Detection of the β-gal expression was described previously [20, 21]. Briefly, samples of the kidney from rats and mice were embedded in OCT compound (Miles Laboratories, Elkhart, Ind., USA), frozen in liquid nitrogen, and cut into thin (10–20 μm) sections. The sections were fixed with 0.2% glutaraldehyde for 5 min at room temperature, washed three times in 0.1 *M* of phosphate-buffered saline (PBS; pH 7.4) for 5 min, transferred to X-gal staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside, 2 m*M* $MgCl_2$, 5 m*M* potassium hexacyanoferrate [III], 5 m*M* potassium hexacyanoferrate [II] trihydrate] at 37°C for 2 h, and then counterstained with kernechtrot solution. To detect β-gal expression in cultured cells, the cells were washed with PBS three times, fixed with 0.2% glutaraldehyde for 5 min at room temperature, washed with PBS three times, and reacted with β-gal staining solution.

Immunohistochemistry

Fresh-frozen kidney sections were fixed with 100% acetone for 20 min at room temperature, washed, and transferred to X-gal staining solution as described above. Using a standard avidin-biotin complex technique, the sections stained with X-gal were blocked with 1.5% H_2O_2 for 10 min, 5% bovine serum albumin/5% horse serum for 20 min, and avidin/biotin (Vector Laboratories, Burlingame, Calif., USA) and incubated for 2 h with rabbit anti-rat AQP-1 antibody (dilution 1:1,000), followed by biotin-conjugated antirabbit IgG (Rockland Immunochemicals, Gilbertsville, Pa., USA, dilution 1:250) for 1 h and horseradish peroxidase-labeled streptavidin (Vector Laboratories; dilution 1:200) for 30 min. Immunoreactive cells were detected with 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan). To estimate the transduction efficiency, X-gal-positive cells of AQP-1-positive cells were quantified using a grid point counting method. The percentages of cells double positive for both X-gal and AQP-1 were calculated.

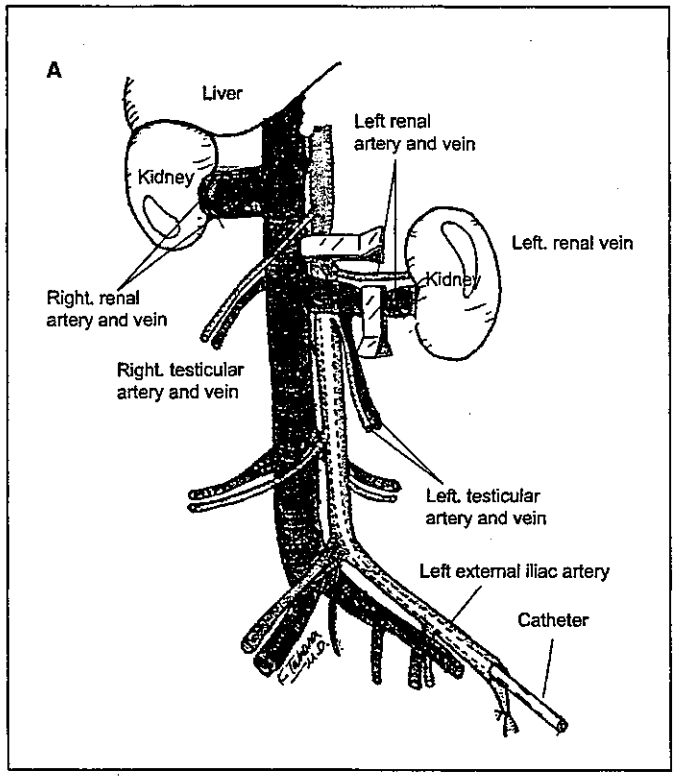

Fig. 1. Procedure for catheter-based gene delivery into the kidney. **A** A 2-Fr flexible catheter is inserted via rat left iliac artery and abdominal aorta, and the tip is placed into the left renal artery. After the aorta is clamped just above the left renal artery, AAV solution is injected after saline to wash out blood through the catheter. The left renal vein then is clamped for 10 min. **B**, **C** The color of the kidney was dark-red before (**B**; arrows) and pale after (**C**; arrows) injection of AAV solution into mice.

In vitro Gene Transduction in NRK52E Cells

The cells were plated on 24-well dishes (Falcon) at 5×10^4/well. After incubation for 16 h, 1.5×10^{10} vector genomes of each of the AAV serotype 1–5 vectors were added to each well (3×10^5 particles/cell). The cells were cultured for 48 h, and the β-gal gene expression was evaluated histologically.

In vivo Gene Transduction into the Kidney

Gene delivery was performed with each of the AAV serotype 1–5 vectors (5.0×10^{11} and 1.0×10^{11} vector genomes in rats and mice, respectively). To achieve efficient gene delivery into the kidney in vivo, we developed a catheter-based gene delivery system for use in rats and mice that mimicked the clinical procedure. The flexible Solo-Cath catheter (2 and 1 Fr for rats and mice, respectively; Solomon Scientific, Plymouth Meeting, Pa., USA) was inserted via the left iliac artery and the abdominal aorta, and the tip was placed beneath the left artery (fig. 1A). After the aorta was clamped just above the left renal artery, 1 ml (rats) or 0.5 ml (mice) of AAV vector solution was injected after 1–2 ml of saline to wash out blood via the catheter. The left renal vein then was clamped for 10 min. Catheter and clip were removed. Figures 1B and C show the color of the kidney before and after the injection of AAV vector solution into the left kidney of mice. The color of the injected kidney changed from dark-red to pale. Histological analysis confirmed that no injury occurred

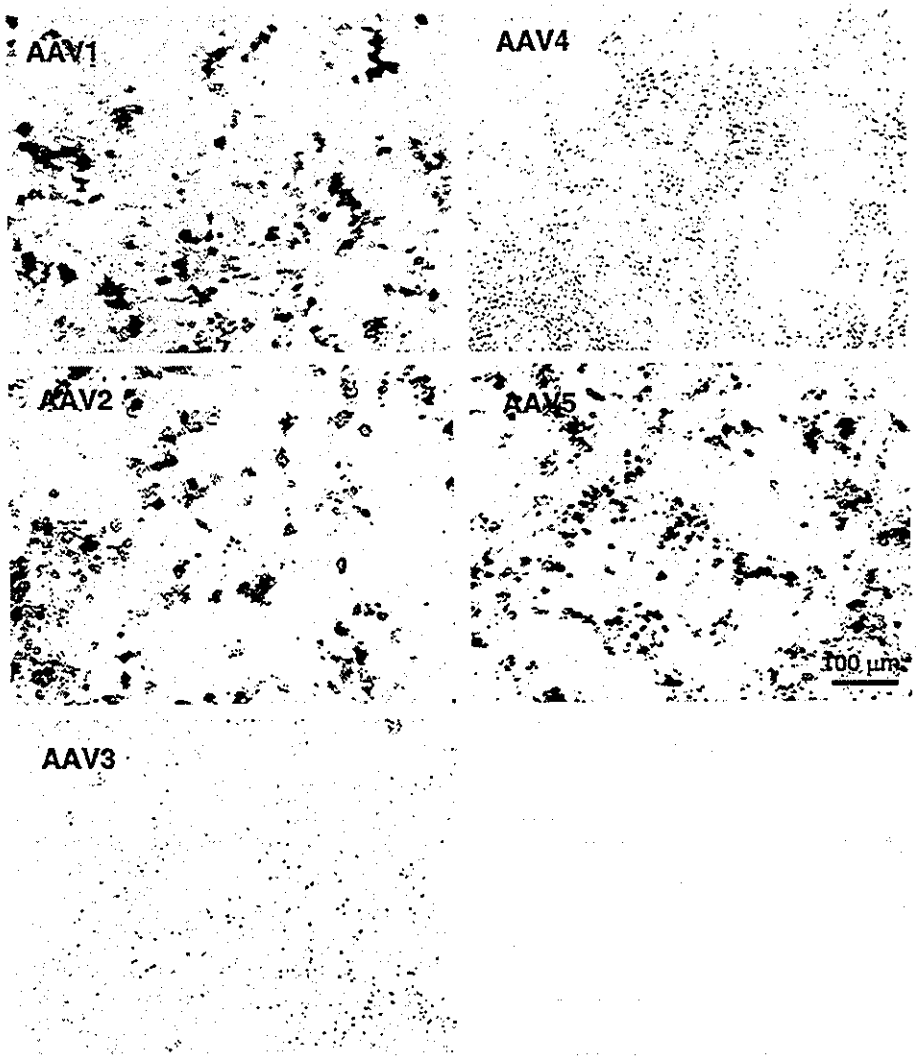

Fig. 2. In vitro transduction of NRK52E cells by AAV serotype derived vectors. NRK52E cells were transduced with AAV serotype 1–5 vectors for 48 h, and the β-gal expression was evaluated. Efficient gene transduction is observed in the cells transduced with AAV serotype 1, 2, and 5 vectors. The results are representative of two independent experiments.

in associationwith this procedure in the injected kidney. Each serotype of AAV vectors was delivered into 2 rats and mice each which were sacrificed on day 14 to evaluate which serotype of AAV vectors had the ability for gene transduction. To investigate long-term gene transduction, additional 2 rats were transduced by AAV2 vectors and sacrificed on day 28.

Results

In vitro Transduction by AAV Serotype Derived Vectors in NRK52E Cells

To evaluate the efficiency of AAV serotype derived vectors for gene transfer into renal cells, we used rat epithelial cell line NRK52E cells as an in vitro model. The cells were exposed to AAV serotype derived vectors encoding the β-gal gene for 48 h, and the β-gal expression was evaluated. The AAV serotype 1, 2, and 5 vectors efficiently transduced these cells (fig. 2). These observations suggest that AAV vectors are useful for gene delivery in renal cells in vitro and that the transduction efficiency might differ among AAV serotypes.

In vivo Gene Transduction by Distinct AAV Serotype Derived Vectors into the Kidney

The vector solutions were selectively injected into rat kidneys using the procedure as described in Materials and Methods, and the animals were sacrificed and the β-gal expression evaluated 14 days after gene delivery. The injection of AAV serotype 2 vectors showed β-gal expression, whereas the rest of the serotypes showed no expres-

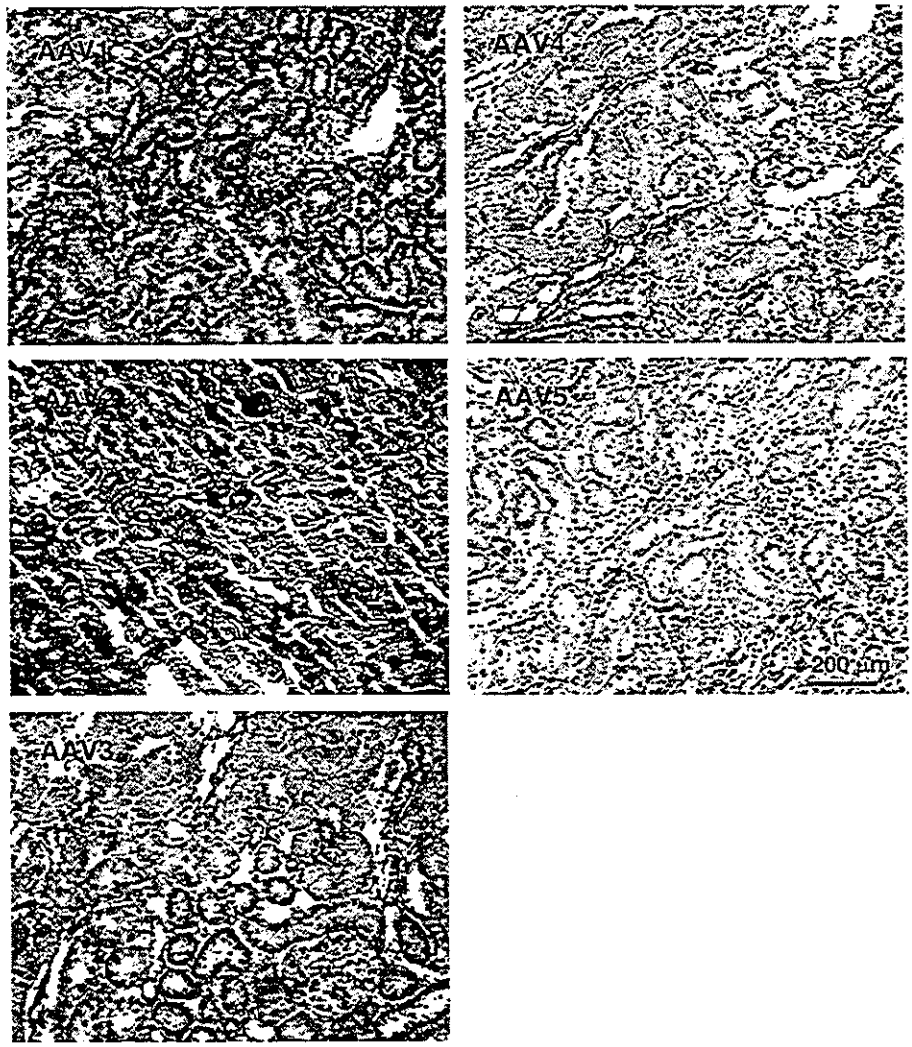

Fig. 3. In vivo transduction by AAV serotype derived vectors into the rat kidney. Solutions with AAV serotype 1–5 vectors encoding the β-gal gene were selectively injected into rat kidney. The animals were sacrificed on day 14, and the kidney sections were evaluated for β-gal expression. Injection of AAV serotype 2 vectors resulted in gene transduction, whereas AAV serotype 1, 3, 4, and 5 vectors showed no transduction. The results represent two independent experiments.

sion (fig. 3). Transduction was observed in cortical tubular epithelial cells, but not in glomeruli, endothelial cells, smooth muscle cells, or interstitial cells. We further confirmed the transduction efficiency with AAV serotype derived vectors into the murine kidney. Consistent with the experimental findings in rats, only AAV serotype 2 vectors showed β-gal expression in the tubular epithelial cells in the kidneys of mice, whereas the other serotypes showed no expression (fig. 4).

We next evaluated the long-term gene transduction by AAV serotype 2 vectors and demonstrated β-gal expression in the rat renal cortex 28 days after gene delivery (fig. 5A). No β-gal expression was observed in extrarenal organs, including liver or skeletal muscle. Immunohistochemical analysis revealed coexpression of β-gal with a proximal tubule marker, AQP-1 [22], suggesting AAV serotype 2 vector mediated gene transduction into the proximal tubular cells. The proportions of gene transduction were 3.2 and 4.0% of the proximal tubular cells.

Discussion

The two major findings of this study are that, in vitro, the AAV serotype 1, 2, and 5 vectors transduced gene expression in kidney epithelial cells, whereas AAV serotype 3 and 4 vectors showed no transgene expression; in vivo, only the AAV serotype 2 vectors transduced gene expression in the proximal tubule of kidney when the vectors were selectively injected into the kidney of rats and

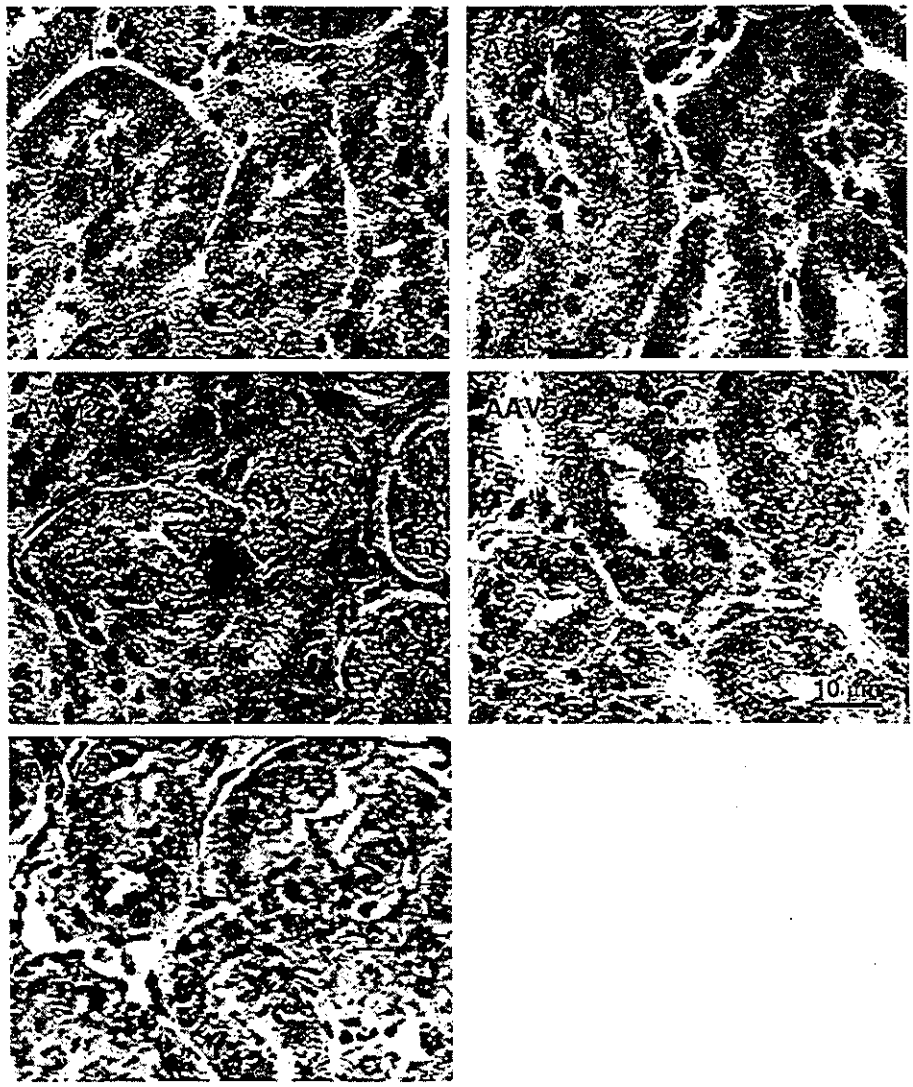

Fig. 4. In vivo gene transduction by distinct AAV serotype vectors into the mouse kidney. Transduction of AAV vectors and evaluation of β-gal expression in mice are essentially described in the figure 1 legend. Consistent with the experiments in rats, the injection of AAV serotype 2 vectors showed gene transduction in the kidney of mice, whereas AAV serotype 1, 3, 4, and 5 vectors showed no transduction. The results are representative of two independent experiments.

mice using a catheter-based procedure. These findings suggest that the transduction efficiency might differ among AAV serotype vectors and that kidney-specific gene delivery using the AAV serotype 2 vectors has the potential for renal gene therapy. The difference in the relative transduction efficiency was not due to a difference in the potency of the serotype-derived vectors, because the capacity of each of the vector stocks was tested and confirmed in vitro and in vivo (data not shown).

Gene transfer into the kidney has a great potential as a novel therapeutic approach. However, efficient gene transduction to the kidney has not been established. Adenovirus-mediated gene delivery is a feasible strategy for gene transduction in the kidney, because it transduces efficient gene expression in the kidney in several experimental models [4–6]. However, there are substantial limitations associated with the clinical application of adenovirus vectors. First, adenovirus vectors show short-term expression (weeks to months) of the transduced gene, because adenovirus does not integrate into the host genome. Second, adenovirus vector mediated gene transduction may cause adverse inflammatory and immunologic responses. Moreover, the risk of recombination with wild-type and generation of replicative mutant virus has not been eliminated.

In contrast to adenovirus vectors, AAV vectors have a number of attractive features for the clinical use of gene therapy. Recombinant AAV vectors are safe, nonpatho-

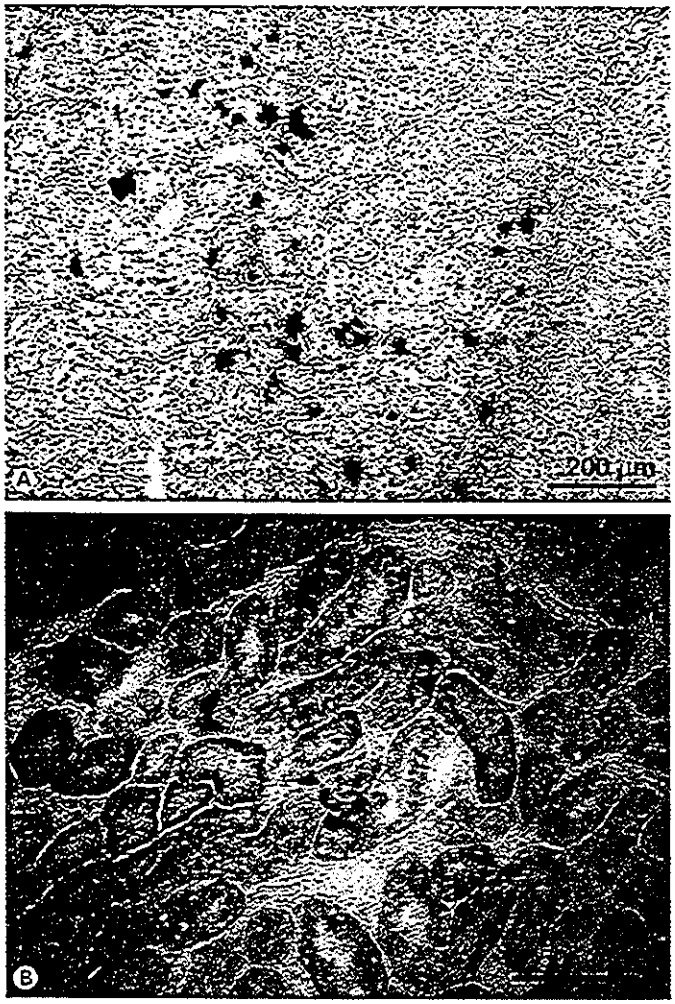

Fig. 5. In vivo long-term transduction by AAV serotype 2 vectors into the rat kidney. Solutions with AAV serotype 2 vectors encoding the β-gal gene were selectively injected into rat kidney, and the rats were sacrificed on day 28. The kidney sections were evaluated for β-gal expression (**A**) and immunohistochemical staining with anti-rat AQP-1 antibody (**B**).

genic, and nonreplicating according to the life cycle of the wild-type virus [23, 24]. Further, unlike the reports using adenovirus vectors, AAV vectors can give rise to long-lasting gene expression without an obvious immune response [9]. An increasing number of reports demonstrated that AAV vectors can transduce into various cell types. Regarding the kidney, the usefulness of the AAV vectors was demonstrated in vitro [25] and in vivo [26]. AAV serotype 2 was used in both studies, because it was representative of AAV and because other serotypes were not focused at that time. However, it is now possible to compare the efficiency of gene transfer into the kidney by using distinct AAV serotype vectors. We, therefore, evaluated the usefulness of AAV serotype 1–5 vectors in renal cells in vitro and in vivo. Interestingly, AAV serotype 1, 2, and 5 vectors transduced renal epithelial cell lines in vitro, whereas only AAV serotype 2 vectors transduced the tubular epithelial cells. Consistent with our findings, AAV serotype 2 vector mediated delivery of reporter genes into tubular epithelial cells was shown in the mouse kidney. Of note, only the tubular epithelial cells, but not the glomerular or vascular cells, were transduced following in vivo AAV serotype 2 vector administration. This means that the tubular epithelial cells are highly susceptible to AAV serotype 2, but the reason for this phenomenon is unclear. Thus far, heparan sulfate as a primary receptor [27] and fibroblast growth factor receptor [28] and αvβ5 integrin [29] as coreceptors have been identified for AAV serotype 2. Therefore, the distribution of these molecules might explain the difference in transduction efficiency. Further investigations are required to determine the precise mechanisms of AAV-mediated gene transfer and to promote gene therapy approaches using AAV vectors in the kidney.

To establish a safe and clinically relevant approach, we developed the catheter-based method of AAV vectors to achieve an organ-specific gene delivery. Alternative approaches for kidney gene delivery have been also demonstrated. Lipkowitz et al. [26] reported that intraparenchymal injection of AAV serotype 2 vectors in the mouse kidney could transduce into tubular epithelial cells. Furthermore, successful gene delivery from renal vein or ureter into the kidney using naked DNA or HVJ liposomes was reported as other approaches for kidney-targeted gene transfer [2, 30, 31]. Since the approach developed in this study mimicked the clinical procedure and might be more physiological than alternative approaches, we believe that this procedure could be useful for future clinical applications.

In summary, we demonstrated that AAV vectors could transduce β-gal gene expression in renal cells in vitro and in vivo. In particular, the catheter-based direct gene delivery of AAV serotype 2 vectors caused successful gene transduction into the kidney tubular epithelial cells of rats and mice. The procedure demonstrated in this study has clinical advantages, because it seems to allow organ-specific gene delivery in the kidney. Since kidney-specific gene delivery via catheterization of the renal artery is highly feasible in humans, our findings provide useful information for promising strategies in renal gene therapy.

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