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Research Paper

Effect of Co-administration of Tacrolimus on the Pharmacokinetics of Multiple Subcutaneous Administered Interferon-Alpha in Rats

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Purpose. Repeated administration of exogenous proteinic compounds triggers the production of specific antibodies. This reaction is limits not only pharmacokinetic studies in animal but also development of human or humanized proteins as drugs. We investigated the effect of co-administration of tacrolimus on pharmacokinetic of human interferon-alpha (h-IFN) following multiple subcutaneous administration in rats.
Methods. h-IFN was administered at a dose of 5 million IU/kg. For some experiments, tacrolimus was also either subcutaneously or intravenously injected in rats at a dose of 0.001 or 0.5 mg/kg as well as with administration of h-IFN.
Results. Multiple administration of h-IFN without co-administration of tacrolimus induced IgG response at 2 or 3 weeks following first administration in the short dosing interval (daily, once per 3 days, or once per a week), irrespective of the dosing interval. Both intravenous and subcutaneous administration of tacrolimus (0.5 mg/kg) with multiple h-IFN injections successfully suppressed IgG response against h-IFN. Interestingly, in lower doses (0.001 mg/kg), intravenous co-administration of tacrolimus showed much stronger suppressive effect than subcutaneous co-administration.
Conclusion. Intravenous co-administration of tacrolimus (0.001 mg/kg) may be a promising way to assess crossover pharmacokinetic study of human or humanized proteinic formulations with multiple dosing schedules in an experimental animal.

KEY WORDS: antibodies; crossover study; interferon-alpha; pharmacokinetics; tacrolimus.

INTRODUCTION

Proteins which may have therapeutic effects, such as cytokines, hormones and enzymes, have been thought to have potential in alleviating various untreated diseases. Recent advances in molecular biology, such as recombinant DNA technology, provide a great opportunity to design and develop protein-based drugs for clinical use. In addition, there is an increased understanding on the role of proteins belonging to advances in molecular biology. Consequently, recombinant therapeutic proteins, synthetic vaccines, and monoclonal antibodies have become an important class of therapeutic agent (1).

Generally, crossover study, in contrast with parallel study, has the advantage of individual differences and number of experimental animals being minimized, and have been extensively carried out for comparative PK evaluation of various formulations. In a crossover study, each experimental animal receives same number of drug formulations (reference one and new formulations) or receives same proteinic drug, with appropriate wash-out period between each administrations. But all therapeutic proteins are exogenous and have the great potential to cause specific antibody production in experimental animal, and form immunological memory. The antibody produced by primary and/or secondary dose affects the PK of subsequent doses during multiple administrations. In fact, it has been reported that in multiple administration of human interferon omega in cynomolgus monkeys, the PK of a subsequent dose was modulated by antibodies elicited by multiple administration (2). Therefore, to compare the PK of same proteinic drug in the same experimental animal is difficult without methodological improvements.

Immunosuppressants which suppress or prevent immune reactions, are clinically used to prevent allograft rejection

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ABBREVIATIONS: AUC, the area under the serum concentration–time curve; C_{1h}, the serum h-IFN concentration at 1 h after administration; C_{max}, the peak serum concentration; C_{mix}, concentration of h-IFN in the mixture sample; CTL, cytotoxic T lymphocytes; CYP3A4, cytochrome P450 3A4; DDS, drug delivery system; ELISA, enzyme-linked immunosorbent assay; h-IFN, human interferon-alpha; i.m., intramuscularly; i.v., intravenous; mAb, monoclonal antibody; OPD, *o*-phenylenediamine; P-gp, P-glycoprotein; POD, peroxidase; PK, pharmacokinetic; s.c., subcutaneous; T_{max}, the time to reach the C_{max}.

after transplantation (3, 4) and to treat autoimmune diseases such as psoriasis, rheumatoid arthritis and Crohn's disease (5). The calcineurin inhibitors, tacrolimus and cyclosporine A, are widely used as immunosuppressants in the clinic and are known to inhibit T-cell function, although the activity of tacrolimus on immune suppression was 100 times higher than that of cyclosporine A (6). In addition, both immunosuppressants have been shown to inhibit the *in vitro* generation of antibody-secreting cells after stimulation with the B cell activator, pokeweed mitogen, which works in a T cell-dependent manner (7). Therefore, we assume that antibody production following multiple administration of proteinic drug may be suppressed by co-administration with an immunosuppressant, tacrolimus.

In the present study, we investigated the issue of whether co-administration of tacrolimus suppresses specific antibody response against native human IFN (h-IFN) following multiple s.c. h-IFN administration in a rat. Consequently, the co-administration minimized the effect of specific immune responses on PK studies of the proteinic drug. This study may have implications on the development of proteinic formulations and assessment methodologies of their PK in the same animal.

METHODS AND METHODS

Materials

OIF® containing 10 million IU native human IFN (h-IFN, M.W.; 13,000–21,000) in a vial, which is the clinical lyophilized formulation for intramuscular or subcutaneous injection, was obtained from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Prograf®, which is a clinical formulation of tacrolimus for intravenous injection containing 5 mg tacrolimus in 1 mL solution in a vial, was purchased from Astellas Pharmaceutical Co., Ltd. (Tokyo, Japan). All other reagents were analytical grade commercial products.

Animal Experiments

Male Sprague–Dawley rats weighing 170–230 g were purchased from Japan SLC (Hamamatsu, Japan), maintained at 23°C and 60% humidity, and allowed free access to water and standard laboratory chow, which was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The study was performed after approval by our local ethical committee at Otsuka Pharmaceutical Co., Ltd. Injection solution of h-IFN was prepared by the addition of 0.4 mL distilled water to the formulation vial (a final concentration of 25 million IU/mL). Tacrolimus solution was prepared by dilution of an adequate volume of physiological saline to the formulation vial (a final concentration of 0.004 and 2 mg/mL).

The h-IFN solution was subcutaneously administered into the back of rats at a dose of 5 million IU/0.2 mL/kg. For some experiments, tacrolimus was also either s.c. injected into the back of the rat or i.v. injected into the jugular vein of the animal at a dose of 0.001 or 0.5 mg/kg as well as with the s.c. administration of h-IFN. A blood sample (200 μL) was withdrawn from the jugular vein at indicated time points after h-IFN administration. The obtained blood was centrifuged (1,800×*g*, 4°C, 10 min) to obtain a serum sample. All obtained serum samples were stored at −20°C until analysis.

Determination of h-IFN in Serum

Concentration of h-IFN in the serum sample was determined using the sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the method recommended by the manufacturer (Japan Immunoresearch Laboratories. Ltd., Takasaki, Japan). Briefly, 50 μL of h-IFN standard solutions (1.56–100 IU/mL) and the rat serum samples, diluted with buffer included in the assay kit, were applied to each well of a microtiter plate, which was coated with anti h-IFN monoclonal antibody (mAb). The plate was then incubated for 2 h and the wells of a plate were washed five times with wash solution in the assay kit to remove unbound h-IFN. Peroxidase (POD)-linked anti h-IFN mAb solution (100 μL) was applied to each well and the plate was incubated for a further 2 h. To remove POD-linked anti h-IFN mAb, the wells of a plate was washed five times with the wash solution. 100 μl of *o*-phenylenediamine (OPD) containing 0.015% hydrogen peroxide was applied to each well for colorization. After 15 min incubation, the colorization was stopped by addition of 100 μL of 1 N H_2SO_4 solution. The absorbance in each well was determined at 492 nm with a microplate reader (Labsystems Multiscan Bichromatic, Helsinki, Finland). All incubations were performed at room temperature throughout this experiment.

PK Analysis

PK parameters of h-IFN were calculated using non-compartment methods. The peak serum concentration (C_{max}) and the time to reach the C_{max} (T_{max}) were read directly from the serum concentration–time curve from individual animals. The concentration profiles of h-IFN in the serum were analyzed based on the statistical moment theory. The area under the serum concentration–time curve (AUC) was calculated by numerical integration using a linear trapezoidal formula (8).

Determination of h-IFN Specific Antibody

Antibody titer against h-IFN in the rat serum was determined by h-IFN ELISA kit (Japan Immunoresearch Laboratories. Ltd., Takasaki, Japan). Serum samples were diluted ten-fold with buffer from the assay kit. 100 μl of the diluted rat serum was mixed with an equal volume of h-IFN standard solution (100 IU/mL) and then the mixture was incubated for 30 min to allow binding of the anti h-IFN antibodies in the serum to the added h-IFN. After incubation, residual (non-reacted) h-IFN concentration in the mixture was determined using the ELISA kit. Concentration of h-IFN in the mixture sample (C_{mix}) was calculated using a standard curve of h-IFN. Antibody titer against h-IFN in rat serum was defined as the following equation:

$$\text{Antibody titer} = \frac{50(\text{IU/mL}) - C_{mix}(\text{IU/mL})}{50(\text{IU/mL})}$$

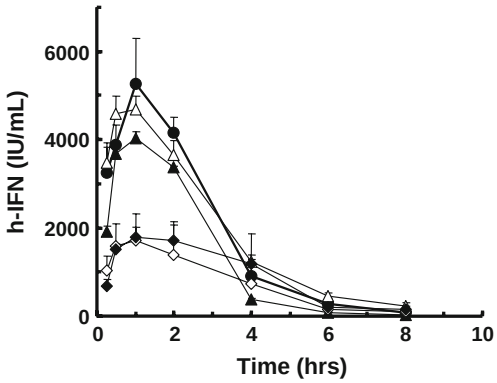

Fig. 1. The serum concentration–time profiles of subcutaneously administered h-IFN at a dose of 5 million IU/kg in rats. *Closed circles* the first dose (day 0), *open triangles* the second dose (day 7), *closed triangles* the third dose (day 14), *open diamonds* the fourth dose (day 21) and *closed diamonds* the fifth dose (day 28). Each value represents the mean ± SE of more than three experiments.

Statistics

Results were expressed as the mean ± SE. Statistical analysis was performed by the Student's *t*-test or Dunnett's test for multiple comparisons, with the minimum levels of significance determined as $p<0.05$.

RESULTS

PK Profiles of h-IFN Following Repeated s.c. Administration

The serum concentration–time profiles of the first [day 0 (control)], second (day 7), third (day 14), fourth (day 21), or fifth (day 28) s.c. administered h-IFN are shown in Fig. 1. The serum concentration of the first (control) or second dose achieved peak at 1 h, then rapidly decreased and disappeared up to 8 h after administration. The serum concentration of the third dose was relatively lower as compared with those of the first (control) and second dose, although the shape of the serum concentration–time profile was similar. In the fourth and fifth doses, much lower serum concentrations (1/2 to 1/3) than those in the first (control), second and third doses were observed.

The PK parameters (AUC_{0-8}, C_{max}, T_{max} and $T_{1/2}$) in each dose were analyzed and summarized in Table I. AUC_{0-8} of the second dose (day 7) was the same as that of first dose (day 0, control). The value of AUC_{0-8} was slightly decreased in the third dose (day 14) and then significantly decreased in fourth or fifth dose (day 21 or 28). The value of C_{max} in each dose showed a similar tendency to AUC_{0-8}. Irrespective of the number of doses, T_{max} was at approximately 1 h after h-IFN administration.

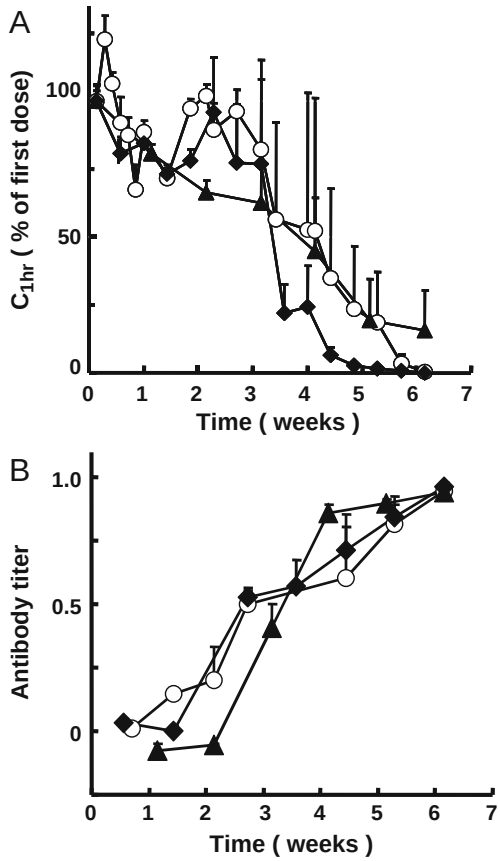

Fig. 2. Effect of dosing interval [daily (*open circles*), once per 3 days (*closed diamonds*) and once per a week (*closed triangles*)] on clearance of h-IFN and immune response against h-IFN in rats. h-IFN was subcutaneously administered at a dose of 5 million IU/kg. **A** $C_{1\,h}$ value of h-IFN (serum h-IFN concentration at 1 h after administration) in each administration. **B** Anti h-IFN antibody titer in the obtained rat serum before subsequent h-IFN administration. Each value represents the mean ± SE of more than three experiments.

Effect of Dosing Interval on h-IFN Clearance and Immune Response Against h-IFN

In order to investigate the effect of dosing interval on the PK profiles, h-IFN was s.c. administered into rats with three

Table I. Pharmacokinetic Parameters of h-IFN after the First, Second, Third, Fourth, or Fifth Subcutaneous Administration of h-IFN at a Dose of 5 Million IU/kg in Rats

Dose (day after the first dose was given)	AUC_{0-8} (IU h/mL)	C_{max} (IU/mL)	T_{max} (h)	$T_{1/2}$ (h)
First (day 0)	14,508±1,687	5,390±964	0.9±0.1	1.0±0.3
Second (day 7)	14,705±880	4,827±330	0.8±0.1	1.5±0.4
Third (day 14)	10,600±81	4,040±155	0.8±0.1	0.9±0.1
Fourth (day 21)	5,932±2,679*	1,742±587 *	1.1±0.3	2.1±2.3
Fifth (day 28)	7,488±2,330*	2,071±399*	1.6±0.8	1.4±0.3

AUC_{0-8} means the area under the serum concentration–time curve of h-IFN from 0 to 8 h obtained following the trapezoidal rule. Each value represents the mean ± SE of more than three experiments
*$p<0.05$, compared with the first dose

dosing schedules (group 1: daily, group 2: once per 3 days and group 3: once per week) for 6 weeks. Because the T_{max} was at approximately 1 h irrespective of dosing schedule (Fig. 1 and Table I), the serum h-IFN concentration at 1 h after administration ($C_{1\ h}$) was measured in each dosing schedule. $C_{1\ h}$ values gradually decreased with the time after the first dose administration, regardless of any dosing group (Fig. 2A). This indicates that the absorption rate of h-IFN became slower and/or the clearance rate became faster at 2 or 3 weeks after administration of the first dose. Titers of anti h-IFN antibodies in the obtained rat sera were also determined. The titers gradually increased after the administration of the first dose, regardless of dosing schedules (dosing groups; Fig. 2B). It appears that there is a clear inverse relationship between the $C_{1\ h}$ value and anti h-IFN antibody-titers, regardless of dosing schedules.

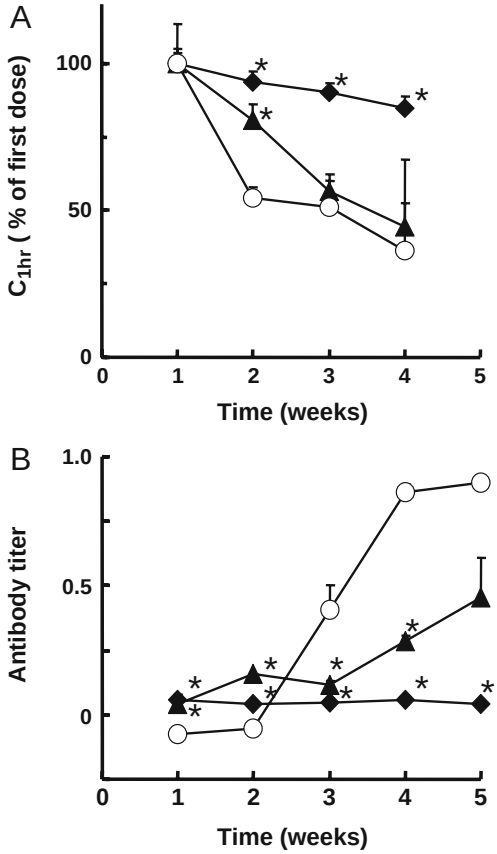

Fig. 3. Effect of subcutaneous co-administration with tacrolimus on PK of h-IFN and immune response against h-IFN in rats. tacrolimus [control: 0 mg/kg (*open circles*), 0.001 mg/kg (*closed triangles*), 0.02 mg/kg (*closed circles*) and 0.5 mg/kg (*closed diamonds*)] was subcutaneously co-administered with h-IFN (5 million IU/kg). h-IFN was also subcutaneously administered. **A** $C_{1\ h}$ value of h-IFN (serum h-IFN concentration at 1 h after administration) in each administration. **B** Anti h-IFN antibody titer in the obtained rat serum before subsequent h-IFN administration. Each value represents the mean ± SE of more than three experiments. *$p<0.05$, compared with control (0 mg/kg).

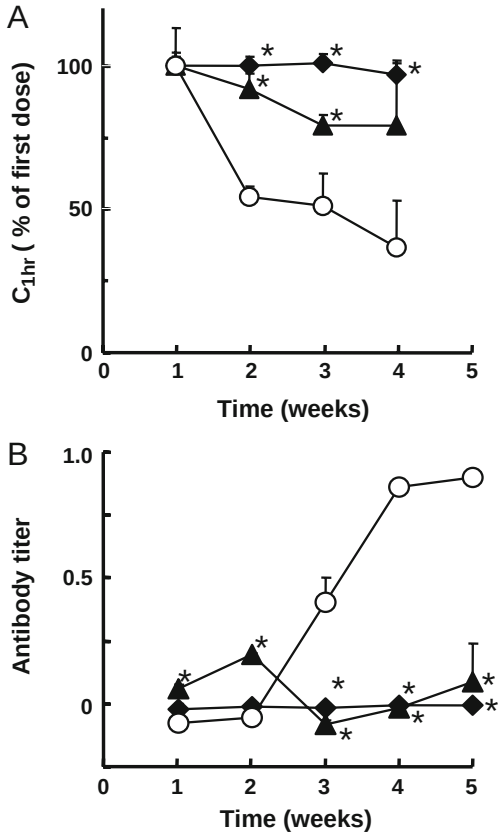

Fig. 4. Effect of the intravenous co-administration with tacrolimus on PKof h-IFN and immune response against h-IFN in rats. tacrolimus [control: 0 mg/kg (*open circles*), 0.001 mg/kg (*closed triangles*), 0.02 mg/kg (*closed circles*) and 0.5 mg/kg (*closed diamonds*)] was intravenously co-administered with h-IFN (5 million IU/kg). h-IFN was subcutaneously administered. **A** $C_{1\ h}$ value of h-IFN (serum h-IFN concentration at 1 h after administration) in each administration. **B** Anti h-IFN antibody titer in the obtained rat serum before subsequent h-IFN administration. Each value represents the mean ± SE of more than three experiments. *$p<0.05$, compared with control (0 mg/kg).

Effect of Co-administration of Tacrolimus on the PK Profile of Multiple Administered h-IFN and Immune Response Against h-IFN

To investigate the effect of co-administration of an immunosuppressant, tacrolimus, on PK of h-IFN, tacrolimus was co-administered with the h-IFN once a week for 4 weeks with two different routes, s.c. or i.v. and three different doses, 0.001 or 0.5 mg/kg.

Without tacrolimus co-administration, the $C_{1\ h}$ value gradually decreased with increasing number of h-IFN dose (Fig. 3A). Subcutaneous co-administrations of tacrolimus inhibited the decrease of $C_{1\ h}$ value of s.c. administered h-IFN in a dose dependent manner. In addition, without co-administration of tacrolimus, the anti h-IFN antibody titers gradually increased with increasing number of h-IFN dose (Fig. 3B). The subcutaneous co-administration of tacrolimus decreased the titers of the antibodies in a dose dependent manner. On the other hand, i.v. administration of tacrolimus, irrespective of doses (0.001 or 0.5 mg/kg), inhibited the decrease of $C_{1\ h}$ value of h-IFN (Fig. 4A). In addition, i.v.

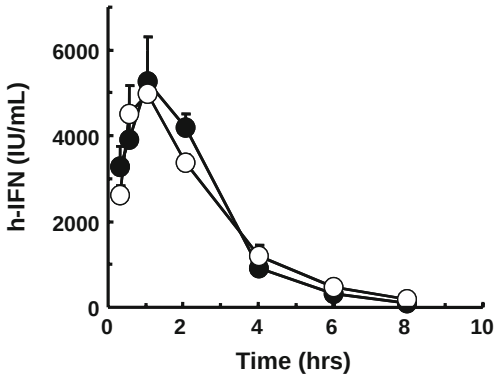

Fig. 5. Effect of intravenous co-administration with tacrolimus on the PK profiles of h-IFN. *closed circles* a single dose of h-IFN (5 million IU/kg), *open circles* co-administration with tacrolimus (0.5 mg/kg). Each value represents the mean ± SE of more than three experiments.

administered tacrolimus inhibited the increase of anti h-IFN antibody titers even at a lowest dose (0.001 mg/kg; Fig. 4B). These findings clearly indicated that co-administration of tacrolimus elicits a common $C_{1\ h}$ value of h-IFN in multiple h-IFN administrations as a consequence of suppression of anti h-IFN antibody production. In addition, tacrolimus administered i.v. shows much higher efficacy than that administered s.c.

The effect of co-administration of tacrolimus on the PK profiles of single dose h-IFN was also determined. Irrespective of co-administration of tacrolimus, similar serum concentration-time profiles were observed (Fig. 5). In addition, there was no significant difference in all obtained PK parameters (Table II).

DISCUSSION

PK profiles of multiple administered h-IFN to the same animal are easily modulated by induced specific antibodies. In this study, we showed that co-administration of an immunosuppressant, tacrolimus, attenuates the modulation of the PK profile of h-IFN after multiple administrations by inhibiting the production of anti h-IFN antibody. In general, a parallel study is carried out for a comparative PK assessment on multiple administration of proteinic drug. However, a crossover study has several advantages compared with a parallel study; it is more statistically worthwhile (9), require a smaller number of animals, and can therefore operate at a lower cost. The results obtained here indicate that a crossover PK study of proteinic drugs could be achieved by co-administration with such an immunosuppressant.

Graft rejection in organ transplantation is caused by $CD8^+$ cytotoxic T lymphocytes (CTL) attacking transplanted tissues (10) and tacrolimus was initially developed as an immunosuppressant for organ transplantation to decrease the chances of graft rejection (11–13). The immunosuppressive effect of tacrolimus on organ transplantation is strongly reflected by the suppression of IL-2 production relating to CTL activation (12). In addition, it has been reported that tacrolimus can suppress the production of IL-4 and IL-5 from T cells (14), which relates to the activation of B cells and differentiation of the B cells into antibody producing plasma cells. We demonstrated that the co-administration with tacrolimus suppressed the production of anti h-IFN IgG in this study (Figs. 3 and 4). Interestingly, the suppressive effect of co-administration of tacrolimus on anti h-IFN antibodies was dependent on the route of administration (i.v. or s.c.) and dose. At a lowest dose (0.001 mg/kg), i.v. administration of tacrolimus showed higher suppressive effect than s.c. administration (Figs. 3 and 4). This may be due to the difference in serum concentration of tacrolimus following injection. Recent studies showed that tacrolimus concentration in blood increased gradually over the first 2 h and was maintained for at least 6 h following s.c. administration of tacrolimus in rats, however the concentration was much lower than that following an intraperitoneal administered dose (15).

Tacrolimus has been reported to cause renal toxicity in several experimental animal species (16–18). Hyperglycemia, hyperkalemia and nephrotoxicity were caused by 3 month i.v. administration of tacrolimus (1 or 2 mg kg^{-1} $week^{-1}$) in rats (16). Nephrotoxicity is the most common side effect of tacrolimus and causes transient increases of serum creatinine and changes in renal function (19, 20). Generally, h-IFN is predominantly eliminated by glomerular filtration, followed by tubular reabsorption and by lysosomal degradation in the kidney (21). Therefore, the PK of h-IFN may be modulated in experimental animals with impaired renal function that is caused by multiple administrations of higher dose tacrolimus. Accordingly, multiple administrations of high dose tacrolimus over long periods must be avoided in a crossover PK study of proteinic drugs predominantly eliminated by glomerular filtration. Intravenous co-administartion of lower tacrolimus dose (0.001 mg/kg) which resulted in complete suppression of the immune reaction against multiple administered h-IFN (Fig. 4) would not cause renal cytotoxicity. I.v. co-administration of tacrolimus with such a lower dose is

Table II. Pharmacokinetic Parameters of h-IFN after a Single Subcutaneous Administration of h-IFN (5 Million IU/kg), With or Without Intravenous Co-administration of 0.5 mg/kg Tacrolimus in Rats

	Pharmacokinetic parameters			
	AUC_{0-8} (IU hr/mL)	C_{max} (IU/mL)	T_{max} (h)	$T_{1/2}$ (h)
h-IFN alone (control)	14,195±1,673	5,390±963	0.9±0.1	1.0±0.2
h-IFN with tacrolimus	15,537±1,690	5,736±283	1.1±0.3	1.3±0.3

AUC_{0-8} means the area under the serum concentration–time curve of h-IFN from 0 to 8 h obtained following the trapezoidal rule. Each value represents the mean ± SE of more than three experiments
There was no significant difference ($p<0.05$) in all obtained PK parameters between h-IFN with tacrolimus group and control group (h-IFN alone)

sufficient to investigate the PK of proteinic drugs in the same experimental animal.

Tacrolimus is a substrate of an efflux transporter, P-glycoprotein (P-gp), and is metabolized by cytochrome P450 3A4 (CYP3A4) (22). Thus there is a possibility that tacrolimus causes drug interactions with other drugs that are also substrates of P-gp or CYP3A4 (23). In this study, we confirmed that the rate of absorption and/or clearance of s.c. administered h-IFN were not affected by i.v. administration of tacrolimus even at a highest dose (0.5 mg/kg; Fig. 5 and Table II). This suggests that our improved strategy is safe and a useful alteration for the assessment of the PK of proteinic formulations. But, it should be noted that there are some reports demonstrating that administration of IFN diminishes expression levels of P-gp *in vitro* (24) and *in vivo* (25). This would reflect an increase of the blood concentration of tacrolimus by h-IFN administration, and thereby cause renal toxicity. Thus, intravenous administration with lower tacrolimus dose such as 0.001 mg/kg (Fig. 5) would be much safer if tacrolimus is to be co-administered with substrates of P-gp or CYP3A4.

CONCLUSION

In the current study, anti h-IFN IgG elicited by multiple administration of h-IFN was suppressed by co-administration of tacrolimus. Interestingly, in the lower doses (0.001 mg/kg), intravenous (i.v.) co-administration of tacrolimus showed much stronger suppressive effect compared with s.c. co-administration. Intravenous co-administration of tacrolimus with a lower dose (0.001 mg/kg), not causing any renal dysfunction, may be a promising way to assess the crossover PK study of human or humanized proteinic formulations with multiple dosing schedule in an experimental animal model.

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