Assessment of Dose Proportionality, Absolute Bioavailability, and Immunogenicity Response of CTLA4Ig (BMS-188667), a Novel Immunosuppressive Agent, Following Subcutaneous and Intravenous Administration to Rats

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Purpose. The objectives of this study were: to delineate the pharmacokinetics of CTLA4Ig in rats after single and multiple intravenous (IV) and subcutaneous (SC) doses; to assess the relationship of the pharmacokinetic parameters of CTLA4Ig vs dose; to calculate the SC absolute bioavailability; and to assess the antibody response of CTLA4Ig.

Methods. A total of 48 (24 male and 24 female) Sprague Dawley rats were divided into eight treatments with 3 rats per gender in each group: a single dose of 10, 80, or 200 mg/kg of CTLA4Ig given either IV or SC and a repeated dose of 10 mg/kg (once every other day for 7 doses over 13 days) given either SC or IV. Serial blood samples were collected up to 43 days after single dose administration and up to 50 days following the administration of the last multiple dose on day 13. The serum concentration of CTLA4Ig and anti-CTLA4Ig antibodies were measured using ELISA assays.

Results. After single IV doses, C_{max} and AUC_{inf} increased in a dose proportional manner; CL appeared to be dose independent, while both V_{SS} and $T_{1/2}$ increased as the administered dose increased. Following single SC doses, C_{max} and AUC_{inf} increased in a linear manner but not proportionally; mean T_{max} values were prolonged but similar among the three dose levels, while $T_{1/2}$ increased as the administered dose increased. The absolute SC bioavailability of CTLA4Ig decreased as the dose increased from 10 (62.5%), 80 (55.7%), and 200 mg/kg (41.1%). Comparison of the AUC_{tau} values between the first and last doses suggested an accumulation (3.1–4.7) of CTLA4Ig. However, regardless of the route of dosing, AUC_{tau} after the last dose were comparable to AUC_{inf} values following the single dose. Anti-CTLA4Ig antibodies were detected at the 10 mg/kg dose level after single or multiple doses for both routes of administration. However, regardless of single or multiple doses, antibody titers were relatively greater for the SC compared to the IV administration.

Conclusions. The key findings of this study were: (i) the elimination characteristics of CTLA4Ig were comparable between the SC and IV routes; (ii) the repeated dosing did not alter the pharmacokinetics of CTLA4Ig; (iii) the SC absolute bioavailability tended to decrease as the administered dose increased; and (iv) a greater formation of anti-CTLA4Ig antibodies was observed after SC compared to IV at a single 10 mg/kg dose level; however, after multiple dosing, the formation of antibodies from either of the two routes was relatively slower, and (v) during the study period, no antibodies were observed at either the 80 or 200 mg/kg dose levels regardless of the route of administration.

KEY WORDS: CTLA4Ig; intravenous; subcutaneous; pharmacokinetics; immunogenicity; rats.

INTRODUCTION

CTLA4Ig (BMS-188667) is a recombinant human fusion protein consisting of the extracellular domain of human CTLA-4 and the Fc region (hinge, CH2 and CH3) domains of the human IgG. It has demonstrated immunosuppressive activity and the ability to induce immunogenic tolerance in several *in vivo* animal models that are associated with T-cell dependent antibody response, i.e. autoimmunity, transplantation, and graft-versus-host-disease (GVHD) (1–6). In a mouse model, suppression of a T-cell dependent antibody response against sheep red blood cells (SRBCs) and keyhole limpet hemocyanin was observed following CTLA4Ig treatment (7). Similar suppression of an antibody response against SRBCs was recently noted in a monkey model following multiple dose treatment with CTLA4Ig (8). The observed suppression of lupus-like disease in NZB/NZW F_1 mice, and partial protection against the occurrence of autoimmune glomerulonephritis in rats following CTLA4Ig dosing demonstrates the utility of CTLA4Ig in autoimmune disease models (9–11). Upon treatment of mice in GVHD models with CTLA4Ig, there was a decrease in the lethal effects of the disease (4,12). The attainment of donor-specific immunogenic tolerance has been reported following CTLA4Ig treatment; induction of tolerance to heart allografts in rats (13), murine skin allografts (14) and vascularized marine cardiac allografts (15). In addition, CTLA4Ig has been shown to promote donor specific tolerance in diabetic mice transplanted with human pancreatic islet cell xenografts (16).

The objectives of the present study were four-fold: (i) to delineate the pharmacokinetics of CTLA4Ig in rats following single and multiple intravenous (IV) and subcutaneous (SC) administration; (ii) to assess the relationship of the pharmacokinetic parameters of CTLA4Ig and the dose administered; (iii) to calculate the SC absolute bioavailability of CTLA4Ig; and (iv) to assess the anti-CTLA4Ig antibody response in rats following SC and IV administration.

EXPERIMENTAL

Study Design

A total of 48 (24 male and 24 female) Sprague-Dawley rats were divided into eight treatment groups with 3 rats per gender in each group: a single dose of 10, 80, or 200 mg/kg of CTLA4Ig was given either IV or SC and a repeated dose of 10 mg/kg (once every other day for 7 doses over 13 days) was given either SC or IV.

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Rat Preparation and Handling

Upon receipt, rats were housed individually in stainless steel, wire-bottom cages of appropriate size and type for the duration of the study. Each rat was tagged with a unique identification number (metal ear tag). Each cage was marked with the same unique number as its occupant with appropriate cage labels. Two days prior to the pharmacokinetic sampling, a jugular catheter was placed in the rat. After each bleed, the patency of the cannula was assured by flushing the cannula with 0.2 ml heparinized-saline solution.

Drug Administration

The dosing solutions contained either 40 mg/ml (SC dosing) or 10 mg/ml (IV dosing) of CTLA4Ig. The dosing solution was sterile filtered using a Sterile Acrodisc^R filter. An appropriate volume of the dosing solution was injected subcutaneously along the dorsal thorax or intravenously into a tail vein using an appropriate size of needle and syringe.

Sample Collection and Preparation

Blood samples (approximately 0.2 ml) were collected up to 43 days following the administration of single doses: predose, and at 1, 2, 4, 8, 24, 48, 96 h, and 8, 15, 22, 29, 36, and 43 days post-dosing. Two additional blood samples were obtained, at 3 and 30 min after IV administration and at 10 and 12 h after SC administration. In a similar fashion, blood samples were collected up to 50 days following the administration of the last multiple dose on day 13. Serum samples were harvested and kept at or below −70°C until analysis.

Analyses of Serum CTLA4Ig

The serum concentration of CTLA4Ig was quantitated using a validated enzyme immunoassay (17). The standard curve concentrations ranged from 2 to 45 ng/ml in rat serum. A 4-parameter logistic regression model of the form, $Y = \max + [(\min - \max)/(1 + (\text{Conc}/ED_{50})^B)]$, was used to fit the data. Prior to the initiation of the study, three sets of quality control (QC) samples, 9, 24, and 38 ng/ml of CTLA4Ig, were prepared in rat serum. The QC samples were stored and analyzed together with the study samples to verify the accuracy, precision, and reproducibility of the assay.

The R^2 values of the standard curves were ≥ 0.994. The mean predicted QC concentrations deviated less than 5.3% of the nominal values. The values for the precision estimates of the QC samples were within 6.7% relative standard deviation. On the basis of the performance of the standard curves and QC samples, the serum assay for CTLA4Ig was accurate, precise and reproducible. The QC data also demonstrated the stability of CTLA4Ig in the rat serum samples during the storage period.

Measurement of Anti-CTLA4Ig Antibodies (Immunogenicity)

Serum titers of antibodies specific for CTLA4Ig were assessed using samples obtained from rats obtained predose and on days 9, 13, 21, 28, 35, 42 for both single and multiple doses. Three additional serum samples obtained on days 49, 56, and 63 following multiple doses were assessed for anti-CTLA4Ig antibody titers. An EIA assay was performed using CTLA4Ig as a capture reagent (plates coated with 2 μg/ml CTLA4Ig) and specific antibody binding was detected using a mixture of commercially available goat anti-rat IgG + IgM specific to heavy and light chains (Jackson Immunoresearch, West Grove, PA) conjugated with alkaline phosphatase as a detection antibody. Prior to detection, serum samples were diluted using a threefold serial dilution scheme starting at an initial dilution of 1:10. Results were represented as end-point titers, where the titer was defined as the reciprocal of the greatest dilution that had an absorbance at least twofold greater than the EIA plate background. An individual rat was judged to have demonstrated a positive immune response against CTLA4Ig when its titer increased by two or more serial dilutions relative to its predose titer.

Pharmacokinetic Analyses

Serum data were subjected to noncompartmental pharmacokinetic analysis (18,19). The following parameters were calculated: peak plasma concentration (C_{max}), time to the attainment of C_{max} (T_{max}), area under the serum concentration-time curve from time = 0 to time = infinity (AUC_{inf}), area under the serum concentration-time curve in a dosing interval (i.e. 48 hours) AUC_{tau}, mean residence time (MRT), terminal serum elimination half-life ($T_{1/2}$), total body clearance (CL) and steady-state volume of distribution (V_{as}). The SC bioavailability (F) was estimated as the quotient of the SC and IV AUC_{inf} values. The accumulation factor was calculated as the quotient of AUC_{tau} values obtained after the last dose and the first dose.

Statistical Methods

The pharmacokinetic parameters after single IV doses (C_{max}, AUC_{inf}, MRT, $T_{1/2}$, CL, and V_{ss}) and SC doses (C_{max}, AUC, T_{max}, MRT, and $T_{1/2}$) were evaluated in the context of an analysis of variance (ANOVA) model. The model included effects for dose, gender, route, gender*dose interaction, gender*route interaction, dose*route interaction, gender*dose*route interaction as well as an error term. If statistically significant (p value < 0.05) interactions were found, all subsequent analyses were performed within the effects involved in that interaction for that parameter. Tukey's unweighted studentized range test was used to make pairwise comparisons among all means for significant model effects as well as to compare the $T_{1/2}$ values between single and multiple doses (20).

Weighted linear regression analysis (1/dose) was performed to evaluate the relationship between C_{max} versus dose and AUC_{inf} versus dose. A test for nonlinearity was performed by testing the model for lack of fit (21). In the absence of significant nonlinearity, the parameter was concluded to be dose proportional if the intercept was not statistically significantly different from zero.

RESULTS

The mean serum concentration data following single IV and SC administration of CTLA4Ig are graphically illustrated

in Figure 1. Comparative graphical representation of single versus multiple dose serum concentration data for IV and SC administration are provided in Figure 2.

Pharmacokinetics After a Single Intravenous Dose

No significant gender effect was observed, therefore, the weighted regression analysis was performed on the pooled data. C_{max}, i.e., the concentration of the drug in the first sample after dosing, and AUC_{inf} values increased as the dose administered increased in a dose proportional manner in rats (R^2 = 0.989). When the doses of CTLA4Ig increased in a ratio of 1:8:20, the C_{max} values increased in a ratio of 1:8.9:18.9 and the AUC values increased in a ratio of 1:7.1:15.6.

CL showed a slight increasing trend with the increasing dose; however, the change was not statistically significantly different. V_{ss} increased significantly as the administered dose

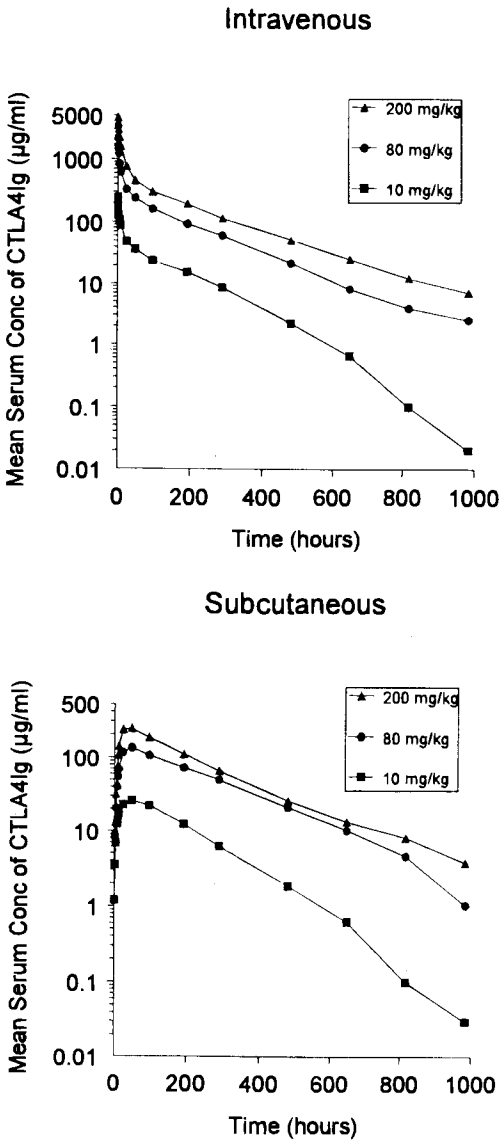

Fig. 1. Mean serum concentrations versus time profiles of CTLA4Ig in rats (N = 6) receiving a single intravenous or subcutaneous dose of CTLA4Ig at the 200, 80, and 10 mg/kg dose levels.

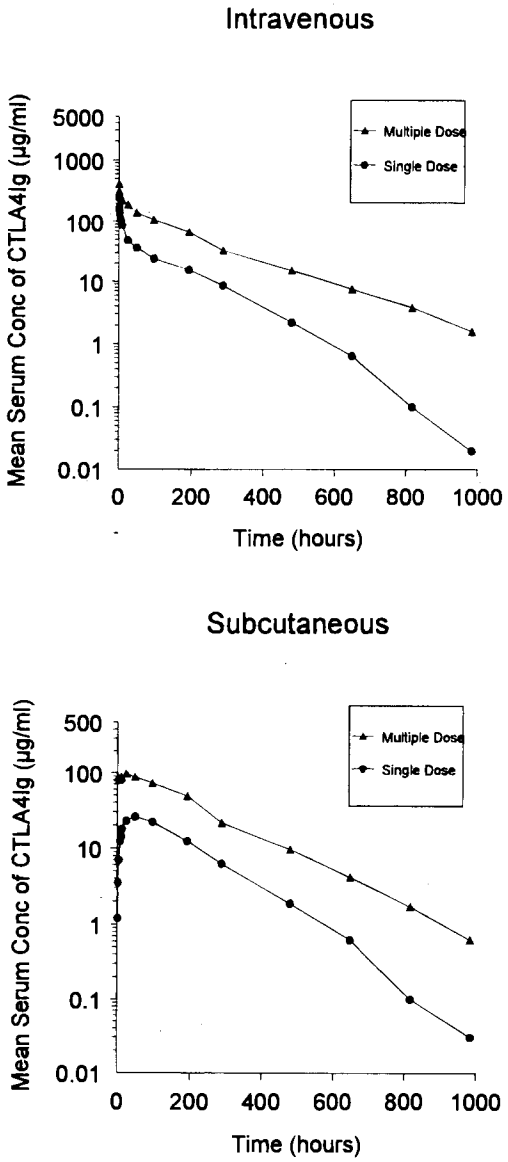

Fig. 2. Mean serum concentrations versus time profiles of CTLA4Ig in rats (N = 6) after single (day 1) and multiple dose (day 13) of CTLA4Ig either intravenously or subcutaneously at the 10 mg/kg dose level.

of CTLA4Ig increased. However, no significant gender effect was observed. Significant differences were observed in the mean MRT values for CTLA4Ig between the male and female rats. The mean MRT values for CTLA4Ig increased as the administered dose increased, however, the increase was not statistically significant. The mean $T_{1/2}$ values for CTLA4Ig increased as the administered dose increased and showed a significant gender*dose interaction. However, there were no gender differences in the mean values of $T_{1/2}$ with one exception, i.e. at the 80 mg/kg dose level the $T_{1/2}$ was statistically significantly longer for the males than the females.

Pharmacokinetics After a Single Subcutaneous Dose

Based on the weighted regression analysis of the pooled data, C_{max} and AUC_{inf} values increased as the dose increased

Table 1. Mean (S.D.) Pharmacokinetic Parameters for CTLA4Ig in Rats (N = 6) After Receiving a Single Intravenous Dose of CTLA4Ig at the 10, 80, and 200 mg/kg Dose Levels

Dose (mg/kg)	C_{max} (μg/ml)	AUC_{inf} (h. μg/ml)	MRT (h)	$T_{1/2}$ (h)	CL (ml/h/kg)	V_{ss} (ml/kg)
10	243.4 (27.0)	8857 (1652)	131 (18.0)	64.3 (20.8)	1.17 (0.26)	149 (15.8)
80	2162 (158)	63167 (9012)	152 (26.7)	108 (55.5)	1.29 (0.16)	193 (21.8)
200	4610 (197)	138608 (23256)	167 (30.6)	170 (29.2)	1.48 (0.26)	243 (32.0)
Statistical[a] comparison	ND	ND	*200 80 10* *m f*	f:*200 80 10* m:*200 80 10* 10: *f m* 80: *m f* 200: *m f*	*10 80 200*	*10 80 200*

[a] Tukey's test (levels not connected with a common underline are statistically significantly different); ND: not determined; f: female; m: male.

in a linear manner in rats (R^2 = 0.937) but not proportionally. When the doses of CTLA4Ig increased in a ratio of 1:8:20, the C_{max} values increased in a ratio of 1:5.1:10.1 and the AUC values increased in a ratio of 1:6.3:10.3.

The absolute SC bioavailability of CTLA4Ig at each dose level decreased as the administered dose increased, 62.5%, 55.7%, and 41.1% at the 10, 80, and 200 mg/kg dose levels, respectively. The mean T_{max} values for CTLA4Ig were 36–48 hours but were similar among the three dose levels; no significant dose or gender effect was observed in the T_{max} values of CTLA4Ig. Significant differences were observed in the mean MRT values for CTLA4Ig between the male and female rats. Similar to the IV administration, the mean $T_{1/2}$ values for CTLA4Ig following SC administration increased as the administered dose increased. There were no gender differences in the mean values of $T_{1/2}$ at the three dose levels. The mean $T_{1/2}$ value for CTLA4Ig following SC route was generally similar to the corresponding value obtained for the IV route.

Table 2. Mean (S.D.) Pharmacokinetic Parameters for CTLA4Ig in Rats (N = 6) After Receiving a Single Subcutaneous Dose of CTLA4Ig at the 10, 80, and 200 mg/kg Dose Levels

Dose (mg/kg)	C_{max} (μg/ml)	T_{max} (h)	MRT (h)	AUC_{inf} (h.μg/ml)	$T_{1/2}$ (h)
10	26.00 (6.13)	48** (24, 96)	165 (29.3)	5536 (589)	74.4 (16.0)
80	133.3 (22.6)	48** (48, 48)	232 (36.3)	35153 (8255)	132 (26.0)
200	262.6 (54.5)	36** (24, 48)	223 (37.9)	56900 (11338)	167 (26.9)
Statistical[a] comparison	ND	NS	*80 200 10* *m f*	ND	f:*200 80 10* m:*200 80 10* 10:*m f* 80:*m f* 200:*m f*

Note: ND: not determined; NS: no significant dose or gender effect was detected in the model. **median (min, max) value reported.
[a] Tukey's test (levels not connected with a common underline are statistically significantly different).

Pharmacokinetics After Multiple Intravenous and Subcutaneous Doses

In rats receiving multiple IV doses, the mean C_{max} value after the last dose was 1.7-fold greater than the C_{max} value on day 1. The comparison of the AUC_{tau} value within a dosing interval (tau = 48 h) between the single and the multiple doses suggested a 3.1-fold accumulation in rats following a once every other day dosing scheme for 7 doses. The mean $T_{1/2}$ value for CTLA4Ig following the multiple dose was significantly greater than that of the single dose.

In rats receiving multiple SC doses, the mean C_{max} value after the last dose was 3.8-fold greater than the C_{max} value on day 1. The comparison of the AUC_{tau} value within a dosing interval between the first and the last doses suggested a 4.7-fold accumulation in rats following a once every other day dosing scheme for 7 doses. The mean $T_{1/2}$ values for CTLA4Ig were longer following the multiple dosing than the single dos-

Table 3. Individual and Mean (S.D.) Pharmacokinetic Parameters for CTLA4Ig in Rats (N = 6) After Receiving Multiple Intravenous and Subcutaneous Doses of CTLA4Ig at the 10 mg/kg Dose Level

Route	C_{max} (μg/ml)	AUC_{tau} (h.μg/ml)	$T_{1/2}$ (h)
Subcutaneous (SC)	99.28 (15.7)	4340 (680)	96.4 (38.4)
Intravenous (IV)	414.8 (31.7)	9412 (1178)	115 (28.5)
Statistical comparison[a]	ND	ND	*SC IV* *m f*

[a] Tukey's test (levels not connected with a common underline are statistically significantly different).

ing; however, statistical differences were only observed in the IV groups.

The mean AUC_{tau} value for CTLA4Ig after multiple (q2d × 7) IV or SC administration at the 10 mg/kg dose level were comparable to the corresponding AUC_{inf} value following the single 10 mg/kg dose, suggesting that the repeated dosing did not alter the pharmacokinetics of CTLA4Ig.

Immunogenicity Response

After single IV or SC dose administration, anti-CTLA4Ig antibodies were detected in the animals receiving a 10 mg/kg dose (Figure 3). Anti-CTLA4Ig antibodies were first detected on day 21 in 17% of the animals receiving CTLA4Ig intravenously and on day 28, in 80% of the animals receiving CTLA4Ig subcutaneously. By days 35 and 42, however, anti-CTLA4Ig antibodies were observed in all animals (100%) in the SC dose group and in 60% of the animals in the IV dose group. The maximum anti-CTLA4Ig antibody titer observed after single 10 mg/kg doses was 21,870 and 7,290, for SC and IV treatments, respectively.

Similar to the observations for single dose, anti-CTLA4Ig antibodies were detected in animals receiving multiple SC and IV doses of CTLA4Ig at the 10 mg/kg dose level (Figure 3). Following multiple SC doses, 33% of the animals had detectable anti-CTLA4Ig antibody levels on day 42 and 83% had detectable titers on day 63. In the multiple IV dose group, one of the five animals had a detectable titer on day 35 and another rat had a detectable titer on day 56 which persisted through day 63. In the animals that showed detectable anti-CTLA4Ig antibodies, the maximum titers were 7,290 and 270, for SC and IV treatments respectively.

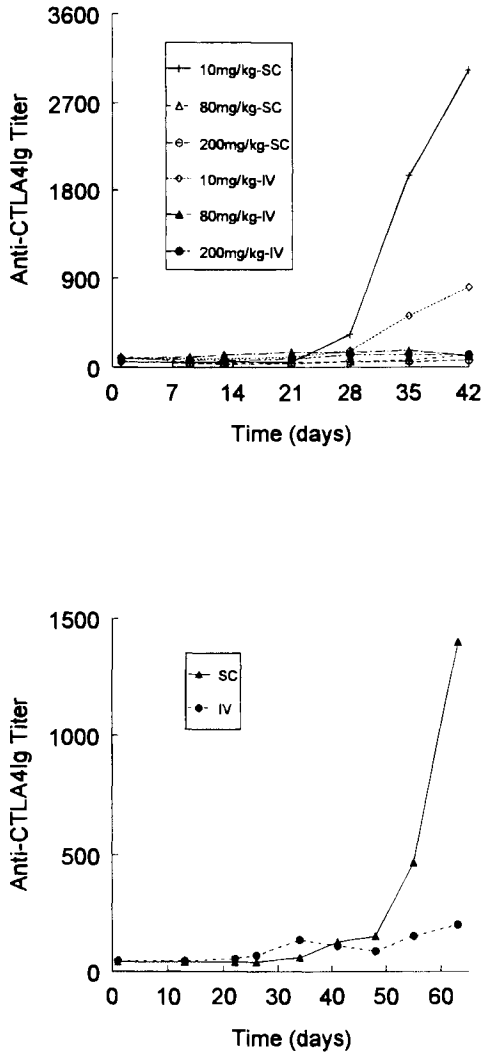

Fig. 3. A plot of Anti-CTLA4Ig titer vs time showing immunogenicity of CTLA4Ig following single (upper panel; 10, 80, and 200 mg/kg dose levels) and multiple (lower panel; 10 mg/kg dose level) subcutaneous and intravenous doses of CTLA4Ig.

DISCUSSION

The pharmacokinetics of several recombinant therapeutic proteins such as human granulocyte-macrophage colony-stimulating factor, human luteinizing hormone, human superoxide dismutase, and human interleukin 3 have been demonstrated to be linear in animals or human subjects (22–25). Likewise, CTLA4Ig, another therapeutic protein, exhibits a dose proportional increase in C_{max} and AUC parameters following single IV doses to mice (3.6–28.6 mg/kg) (26) and monkeys (10–33 mg/kg) (27), and multiple IV doses to monkeys (1–50 mg/kg) (8,27). Consistently, the data obtained from this study demonstrate that both C_{max} and AUC_{inf} increase in a dose proportional manner in rats following a single IV dose (10–200 mg/kg). As observed previously (26,27), CL of CTLA4Ig appeared to be independent of dose, and V_{ss} and $T_{1/2}$ of CTLA4Ig increased as the dose increased.

Similarly to the mice (28), the absorption of CTLA4Ig following SC administration was slow. C_{max} values were several fold lower as compared to those after the IV administration of CTLA4Ig. At the 10 mg/kg dose level, the SC absolute bioavailability for CTLA4Ig was 63%, which was lower than that estimated in mice (85%) (28). Examination of the literature data suggests that the SC bioavailabilities of other therapeutic proteins vary from about 20% to 100%. The SC bioavailabilities of human luteinizing hormone in monkey (23) and human erythropoietin in man (29) were approximately 50% and 20–30%, respectively, while that of the human insulin-like growth factor I was about 100% in man (30). In the present study, the SC bioavailability of CTLA4Ig tended to decrease as the dose administered increased. However, it is not clear what caused the decreased SC bioavailability at the higher dose.

The $T_{1/2}$ values were generally similar between SC and IV routes at each dose level suggesting that the elimination characteristics of CTLA4Ig were unaffected following SC administration. This observation is consistent with the previous finding in mice (24). The shorter $T_{1/2}$ observed after single IV or SC doses as compared to the respective multiple doses is an artifact due to the greater formation of anti-CTLA4Ig antibodies at the single relative to multiple doses.

Recently, antibodies to recombinant human factor IX have been observed in dogs that resulted in the faster elimination of the therapeutic protein (31). In this context, the formation of anti-CTLA4Ig antibodies has been observed previously in both mice and monkeys (8,15,26,28). Consistent with the previous results, at the time of detection of the antibodies, the serum concentration of CTLA4Ig had decreased to approximately

1 μg/ml following the administration of a single 10 mg/kg dose of CTLA4Ig. At higher doses, the sera did not show the presence of anti-CTLA4Ig antibodies presumably because of relatively greater serum concentrations of CTLA4Ig as compared to the 10 mg/kg dose. However, it is very likely that anti-CTLA4Ig antibodies would have been observed at these high doses at a later time point, which would coincide with the decline in serum concentration of CTLA4Ig to approximately 1 μg/ml. The fact that the onset of the anti-CTLA4Ig response was delayed relative to what would be expected for a typical immunogenic protein (31), suggests that CTLA4Ig initially suppressed the antibody response to itself, but that when the serum concentrations fell below an immunosuppressive threshold, anti-CTLA4Ig antibodies were produced. Further support to this hypothesis can be obtained by viewing the multiple dose immunogenicity data. As a result of both longer dosing period and the serum accumulation of CTLA4Ig after multiple dosing, the anti-CTLA4Ig antibody formation was much slower with a lower titer following multiple doses relative to those of the single doses for either of the two treatments. These data also suggest that CTLA4Ig was more immunogenic following SC administration compared to the IV administration in terms of both the incidence of positive antibody formation and in the magnitude of the observed response. This difference may be explained by lower serum concentrations due to the lower bioavailability of CTLA4Ig from the SC administration relative to the IV administration.

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