JPP
Journal of
Pharmacy and Pharmacology

JPP 2001, 53: 727–733
© 2001 The Authors
Received July 13, 2000
Accepted January 10, 2001
ISSN 0022-3573

Comparison of suppressive potency between prednisolone and prednisolone sodium succinate against mitogen-induced blastogenesis of human peripheral blood mononuclear cells in-vitro

Kentaro Sugiyama, Tomie Kawada, Hiroshi Sato and Toshihiko Hirano

Abstract

Clinically, both prednisolone and prednisolone sodium succinate are widely used as immunosuppressive agents for the treatment of various allergic disorders. However, whether prednisolone sodium succinate itself has immunosuppressive or anti-inflammatory effects is unclear, and prednisolone sodium succinate may exhibit its efficacy only after hydrolytic conversion to prednisolone in-vivo. If this is the case, the impairment of prednisolone sodium succinate conversion to prednisolone in some clinical conditions may attenuate the efficacy of prednisolone sodium succinate. We therefore compared the pharmacological efficacy of prednisolone with that of prednisolone sodium succinate in-vitro using human peripheral blood mononuclear cells (PBMCs). PBMCs were obtained from 5 healthy subjects and 1 patient with pneumonia. The cells were incubated in the presence of concanavalin A and the cell growth was estimated by 3-(4,5-dimethyl thiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Both prednisolone and prednisolone sodium succinate dose-dependently suppressed PBMC blastogenesis. Mean (s.d.) prednisolone and prednisolone sodium succinate IC50 (concentration of drug that gave 50 % inhibition of cell growth) values were 580.0 (1037.9) and 3237.1 (4627.3) nM, respectively. The ratio of prednisolone IC50/prednisolone sodium succinate IC50 ranged from 0.005 to 0.230. Thus, prednisolone sodium succinate potency was markedly lower than that of prednisolone. After incubation of PBMCs with 100 μM prednisolone sodium succinate, 22.7–42.9 μM prednisolone was liberated into the culture medium, as determined by HPLC. The ratio of prednisolone liberation from prednisolone sodium succinate was not affected by the presence of fetal bovine serum or PBMC, or both, in the culture medium. These results suggested that the PBMC-suppressive effects of prednisolone sodium succinate might be due, at least partially, to prednisolone liberated from prednisolone sodium succinate into the culture medium. Prednisolone sodium succinate can be converted to prednisolone in the absence of serum or PBMCs, but the ratio of this conversion was very slow (t£frac12; $>$ 4 days). Therefore, impairment of the enzymatic conversion of prednisolone sodium succinate to prednisolone in some pathological conditions such as liver diseases may result in attenuation of the clinical efficacy of prednisolone sodium succinate.

Division of Pharmacy, Niigata University Medical Hospital, 1-754 Asahimatidori, Niigata 951-8520, Japan

Kentaro Sugiyama, Tomie Kawada, Hiroshi Sato

Department of Clinical Pharmacology, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

Toshihiko Hirano

Correspondence: K. Sugiyama, Division of Pharmacy, Niigata University Medical Hospital, 1-754 Asahimatidori, Niigata 951-8520, Japan. E-mail: sugiyama-nii@umin.ac.jp

Introduction

Both prednisolone and prednisolone sodium succinate are widely used for treatment of various allergic disorders as immunosuppressive agents. However, prednisolone has low solubility in water, and therefore it must be esterified to make water-soluble

injectable formulations. Therefore, prednisolone sodium succinate is currently used for intravenous injection. Prednisolone sodium succinate is generally believed to have little or no pharmacological efficacy itself. Prednisolone sodium succinate is known to be hydrolysed to release prednisolone in the body, and the free prednisolone is considered to be effective for immunosuppression (Tamm & Voigt 1960). Therefore, impairment of prednisolone sodium succinate conversion, possibly through enzymatic cleavage of the ester to prednisolone in certain pathological conditions, may attenuate the therapeutic efficacy of prednisolone sodium succinate. However, there have been few reports concerning the immunosuppressive potency of prednisolone succinate itself as compared with prednisolone, or the rate of conversion of prednisolone sodium succinate to prednisolone in biological fluids. If the immunosuppressive effect of prednisolone sodium succinate is due to its parent molecule and is not related to prednisolone sodium succinate itself, the conversion rate of prednisolone sodium succinate to prednisolone in the human body might be critical for its clinical efficacy. Indeed, steroid-ester-metabolizing esterase activity has been reported in human liver, colon, stomach, breast and immune cells (Margaretha et al 1994). This esterase activity may be reduced in certain diseases, and, therefore, the conversion rate of prednisolone sodium succinate to prednisolone may be attenuated in these conditions.

In this study, we compared the pharmacological potencies of prednisolone and prednisolone sodium succinate in-vitro using peripheral blood mononuclear cells (PBMCs) obtained from healthy subjects and from a patient with pneumonia using a mitogen-assay procedure (Hirano et al 1994, 1997b). We also examined the amounts of prednisolone released from prednisolone sodium succinate into culture medium in relation to the suppressive potency of prednisolone sodium succinate on PBMC-blastogenesis.

Materials and Methods

Drugs

Most of the chemicals were obtained from Wako Chemical Co. (Osaka, Japan), except for the following. MTT, prednisolone and prednisolone sodium succinate were from Sigma Chemical Co. (St Louis, MO). Ficoll-Paque was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). RPMI 1640 medium, fetal bovine serum, and HBSS were obtained from Gibco Laboratories (Rockville, NY). Concanavalin A was from Seikagaku Kogyo Co. (Tokyo, Japan). All other reagents were of the highest grade available.

Subjects

After informed consent was obtained, heparinized venous blood (15 mL) was taken from five healthy subjects, aged 22–30 years, and from one patient with pneumonia (aged 76 years). These subjects had no history of taking immunosuppressive agents, including glucocorticoids. To measure the response of PBMCs to drugs in-vitro, the drug sensitivity test (Hirano et al 1994, 1997b) was carried out in each subject as described below.

Isolation of PBMCs

Isolation and culture of PBMCs was carried out according to the method described previously (Hirano et al 1994, 1997b). In brief, heparinized blood was loaded onto 12 mL of Ficoll-Paque and centrifuged at 900 *g* for 20 min at room temperature. The buffy coat was taken, and then rinsed 3 times with Hank's balanced salt solution. PBMCs including lymphocytes were suspended in RPMI 1640 medium containing 10% fetal bovine serum to a cell density of 1×10^6 cells mL^{-1}.

PBMC culture and evaluation of drug potency

The cell suspensions (prepared as above) were placed into each well of microplates with 96 flat-bottomed wells. Saline containing concanavalin A was added to each well to a final concentration of 5.0 $\mu g\ mL^{-1}$. Subsequently, ethanol solution containing prednisolone or an aqueous solution of prednisolone sodium succinate was added to give a final drug concentration of 0.01, 0.1, 1, 10, 100, 1000, 10000 or 100000 nM. The same volume of ethanol was added to control wells (2% ethanol concentration). The plates were incubated for 4 days fumed in 5% CO_2 at 37°C.

MTT assay

After 4 days of culture, 5 mg mL^{-1} 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dissolved in saline was added to each well and then reincubated under 5% CO_2 at 37°C for 4–5 h (Hirano et al 1995). The plates were centrifuged at 375 *g* for 5 min to precipitate cells and formazan produced by growing cells. Samples of the supernatant were removed from each well and dimethylsulfoxide (DMSO) was added, followed by shaking the plate on a microshaker for 10 min to dissolve the formazan crystals. The wells were

read with a microplate reader at 550 nm. Dose–response curves were plotted, and the concentrations of drug that gave 50 % inhibition of cell growth (IC50) were calculated.

Chromatographic measurement of prednisolone

After incubation with 100 μM prednisolone and 100 μM prednisolone sodium succinate for 4 days, the prednisolone concentration in the PBMC culture supernatant was measured by reversed-phase HPLC. The supernatant was subjected to a solid-phase extraction procedure through a bond elute C18 column, and the material retained in the column was eluted with ethanol and H_2O. The eluate was concentrated, and then injected into the HPLC apparatus. Our HPLC system, an LC-3A (Shimadzu, Tokyo, Japan), consisted of a pump, spectrophotometric detector, system controller auto-injector and recorder. Detection wavelength was 260 nm. We used a Shim-pack VP-ODS column (4 mm i.d. $\times$ 250 mm) (Shimadzu, Tokyo, Japan) with a pre-column for analysis of the drugs. The mobile phase was a mixture of water–methanol (4:6; v/v). The flow rate of the mobile phase was 1.0 mL min^{-1}. The data were analysed using the absolute calculation curve method.

Statistics

Correlations between the pairs of indices, prednisolone IC50, prednisolone sodium succinate IC50, and amounts of prednisolone liberated into the medium were analysed using Pearson's correlation coefficient test. Differences between prednisolone IC50 and prednisolone sodium succinate IC50 in Tables 1 and 2 were analysed by Mann-Whitney *U*-test. Differences in mean $\pm$ s.d. values of prednisolone liberated into the medium under different experimental conditions (Figure 4) were compared by a multiple comparison (Dunnett's) test. *P* values of < 0.05 were considered significant.

Results

The effects of prednisolone and prednisolone sodium succinate on in-vitro blastogenesis of PBMCs from subjects were compared. Typical dose–response curves of prednisolone and prednisolone sodium succinate on the mitogen-induced blastogenesis of PBMCs obtained from one healthy subject are shown in Figure 1. Both of the agents suppressed blastogenesis dose dependently, but the effect of prednisolone sodium succinate was markedly weaker than that of prednisolone. The IC50 value of prednisolone sodium succinate was 351.7 nM, which was 4.4-fold higher than that of prednisolone (79.7 nM).

To confirm the differences in individual suppressive effects on PBMCs between prednisolone and prednisolone sodium succinate, we compared the in-vitro effects of these agents using PBMCs obtained from six subjects (5 healthy subjects and 1 patient with pneumonia) (Table 1). The range of prednisolone IC50 values showed large deviations from 9.5 to 2882.0 nM among the subjects. The difference in IC50 was 303.4 fold. The mean (s.d.) prednisolone IC50 was 580.0 (1037.9) nM. Prednisolone sodium succinate IC50 also showed large individual differences ranging from 330 to 13 500 nM (40.9-fold difference), and the mean (s.d.) of these values was 3237.1 (4627.3) nM. No statistically significant correlation was observed between prednisolone and prednisolone sodium succinate IC50 values. We also exam-

Table 1 Comparison of immunosuppressive potency between prednisolone and prednisolone sodium succinate and amounts of prednisolone in PBMC culture medium in five healthy subjects and one patient with pneumonia.

PBMC donors	IC50 value (nM)			Prednisolone detected in culture medium (μM)
	Prednisolone	Prednisolone sodium succinate	Ratio (prednisolone/ prednisolone sodium succinate)	
1	79.7	351.7	0.230	36.2
2	405.0	2181.8	0.190	34.1
3	42.2	1371.0	0.030	42.9
4	2882.0	13500	0.210	33.2
5	9.5	1905.0	0.005	22.7
6[a]	61.7	330.0	0.190	34.3
Mean ± s.d.	580.0 ± 1037.9	3237.1 ± 4627.3	0.140 ± 0.090	33.9 ± 0.09

Table 2 Day-to-day variations in IC50 values for prednisolone and prednisolone sodium succinate in one healthy subject.

No. of experiment	IC50 value (nM)		
	Prednisolone	Prednisolone sodium succinate	Ratio (prednisolone/ prednisolone sodium succinate)
1	79.7	351.0	0.23
2	43.9	161.0	0.27
3	80.8	387.6	0.21
4	43.6	155.0	0.28
5	252.2	1619.3	0.16
6	179.2	780.9	0.23
Mean ± s.d.	113.2 ± 76.9	575.8 ± 510.8*	0.23

Each experiment was carried out on a different day.
*$P < 0.05$ compared with prednisolone IC50 (Mann-Whitney *U*-test).

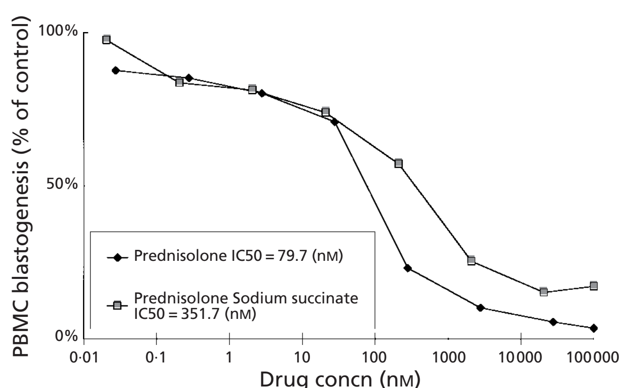

Figure 1 Comparison of dose–response curves between prednisolone and prednisolone sodium succinate on mitogen-induced blastogenesis of PBMCs from one healthy subject.

ined the blastogenesis-suppressing potency of these agents in PBMCs from a patient with pneumonia; the prednisolone IC50 of this patient was within the range of IC50s of these agents obtained with healthy PBMCs. The ratio of prednisolone IC50 to prednisolone sodium succinate IC50 ranged from 0.005 to 0.230, and thus the mean potency of prednisolone was about 7-fold higher than that of prednisolone sodium succinate, although the difference between the IC50 of prednisolone and that of prednisolone sodium succinate was not statistically significant.

Table 2 shows the day-to-day variation of PBMC sensitivities to the drugs in one healthy subject; prednisolone and prednisolone sodium succinate IC50s measured on six different days are presented. The range of prednisolone IC50 values deviated from 43.6 to 252.2 nM (a 5.8-fold difference) with a mean (s.d.) of 113.2 (76.9) nM. Prednisolone sodium succinate IC50 was significantly higher than that of prednisolone ($P <$ 0.05). The range of prednisolone sodium succinate IC50s also deviated widely (155.0–1619.3 nM; a 10.4-fold difference). The ratio ranged from 0.16 to 0.28 (a 1.8-fold difference). These values were small compared with the ratios shown in Table 1. Thus, both the prednisolone and prednisolone sodium succinate IC50 showed large deviations, but the day-to-day variations were small as compared with those of interindividual (subject-to-subject) deviations (Table 1).

The suppressive efficacy of prednisolone sodium succinate on PBMC blastogenesis in-vitro might be at least partially due to prednisolone liberated from prednisolone sodium succinate in culture. Therefore, we examined free prednisolone levels in PBMC cultures incubated in the presence of 100 μM prednisolone sodium succinate as well as in the presence of 100 μM prednisolone. Figure 2 shows prednisolone peaks (obtained by HPLC analysis) in these cultures of PBMCs obtained from one healthy subject after 4 days of culture. In this case, the prednisolone peak in the culture incubated with prednisolone sodium succinate was approximately less than half that in a culture incubated with prednisolone itself. Figure 3 shows a typical time course of changes in prednisolone levels in these cultures. Prednisolone levels in cultures incubated with 100 μM prednisolone were not largely different throughout the culture period (68–80 μM). However, the levels in cultures incubated with prednisolone sodium succinate increased gradually from 16.7 to 36.2 μM from day 1 to day 4. The amount of prednisolone was measured in

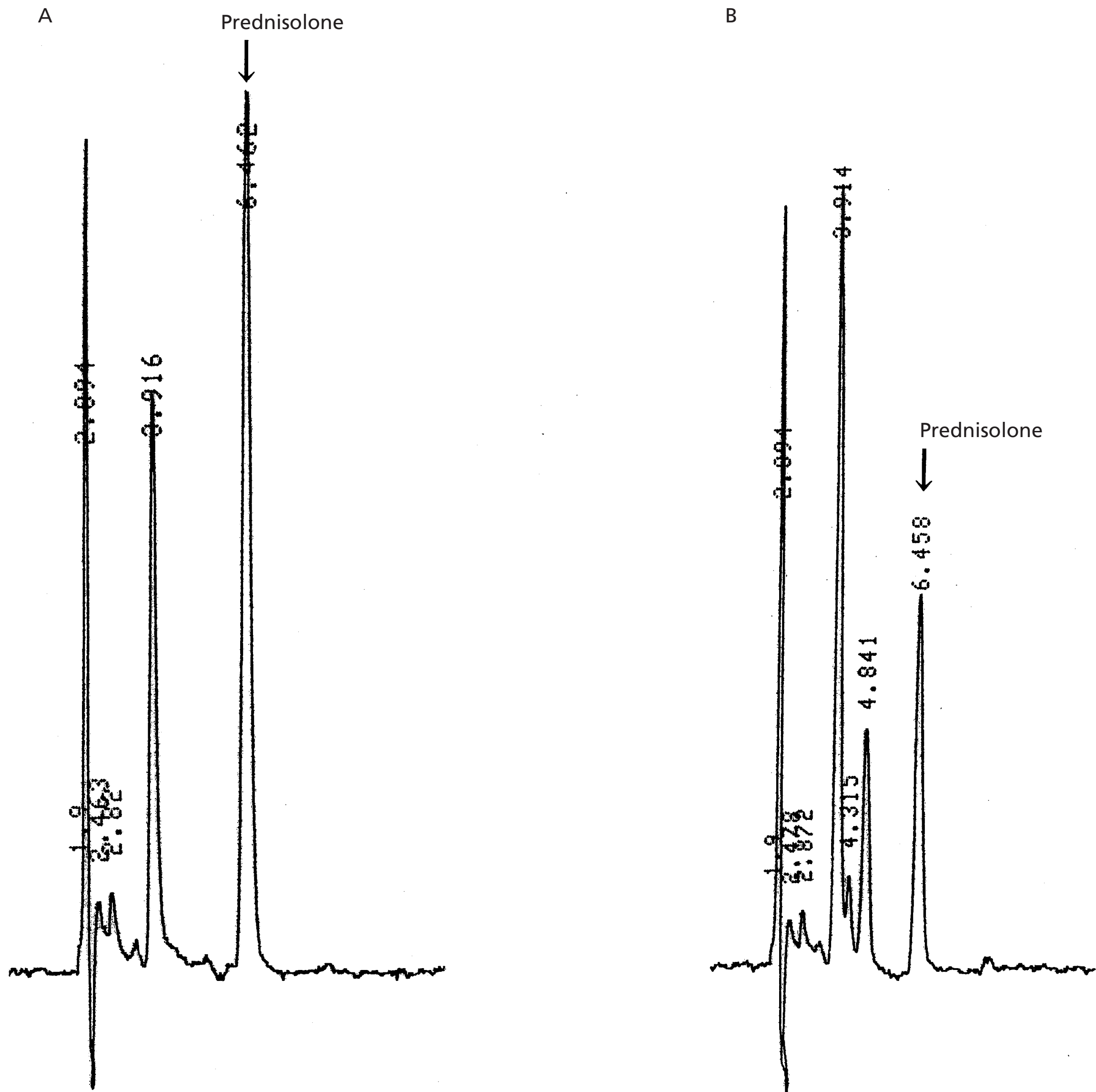

Figure 2 Prednisolone peaks, from HPLC analysis, in cultures of PBMCs from one healthy subject after incubation with 100 μM prednisolone (A) or 100 μM prednisolone sodium succinate (B) for 4 days.

cultures of PBMCs obtained from six subjects (including one patient with pneumonia) in the presence of prednisolone sodium succinate (Table 1). The amount of prednisolone released from prednisolone sodium succinate in these PBMC cultures was 22.7–42.9 μM, and thus the amount of prednisolone released was not markedly different between the PBMC donors. Figure 4 shows that addition of PBMCs or fetal bovine serum (or both) to the culture medium did not markedly promote hydrolysis of prednisolone sodium succinate to prednisolone. These results showed that ~43% of prednisolone sodium succinate was hydrolysed in the medium in the absence of the cells or serum.

Discussion

Using our PBMC culture system, we demonstrated that prednisolone sodium succinate shows immunosuppressive potency in-vitro on PBMC blastogenesis. However, the potency of prednisolone sodium succinate was several fold lower than that of prednisolone. In all PBMC

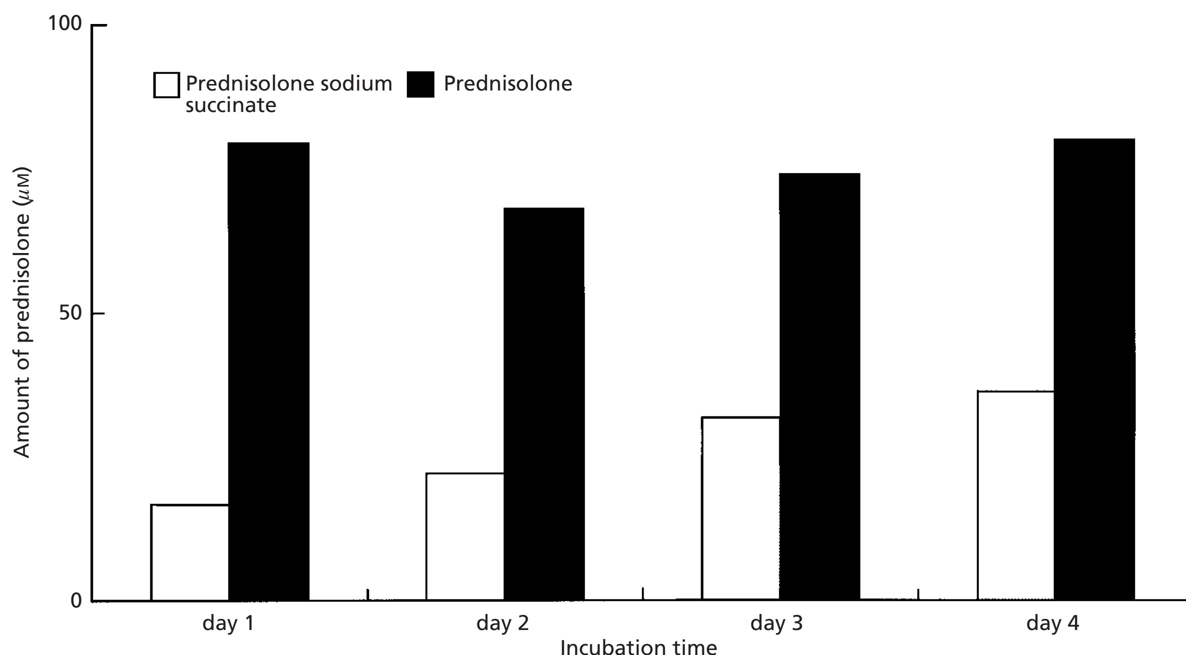

Figure 3 Amounts of prednisolone in PBMC cultures after incubation with 100 μM prednisolone sodium succinate or 100 μM prednisolone for 1–4 days. Representative data from one healthy subject are shown.

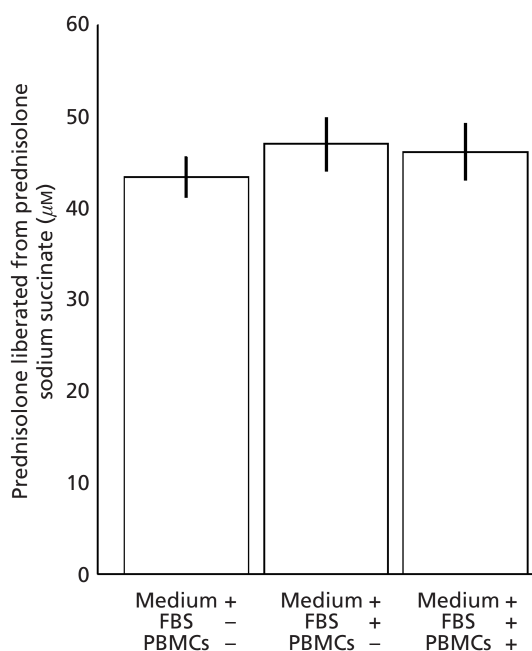

Figure 4 Comparison of amounts of prednisolone hydrolysed from prednisolone sodium succinate in culture medium in the presence or absence of PBMCs and fetal bovine serum (FBS). Results are presented as means ± s.d. of 6 experiments. No significant differences were observed between values by multiple comparison (Dunnett's).

donors tested here, the IC50 of prednisolone sodium succinate was higher than that of prednisolone, and the potency of prednisolone sodium succinate was approximately 7-fold less than that of prednisolone. Prednisolone sodium succinate is slowly hydrolysed to liberate prednisolone in culture even in the absence of serum or PBMCs, and the prednisolone thus produced was suggested to exhibit a PBMC-suppressive effect in-vitro.

In a general sense, prednisolone sodium succinate can be classified as an ester-form prodrug of prednisolone. Prednisolone sodium succinate is hydrolysed to release prednisolone in the human body or tissue (Tamm & Voigt 1960; Margaretha et al 1994), and the free prednisolone might have pharmacological efficacy. Our results were consistent with the suggestion that the PBMC-suppressive efficacy of prednisolone sodium succinate is due to prednisolone liberated by hydrolysis of prednisolone sodium succinate in the culture medium. Esterified steroids have been reported to be hydrolysed by esterases in the human liver, colon, stomach, breast and lymphocytes (Margaretha et al. 1994). We examined whether bovine serum or PBMCs added to culture medium may have such esterase activity to convert prednisolone sodium succinate to prednisolone. Our results showed that hydrolysis of prednisolone sodium

succinate in the culture medium to produce free prednisolone was not markedly promoted by addition of fetal bovine serum or PBMCs (or both) in-vitro (Figure 4). These observations also raised the possibility that the immunosuppressive efficacy of prednisolone sodium succinate might be attenuated in certain pathological conditions, including liver diseases, in which impairment of enzymatic hydrolysis of the steroid ester might occur.

We reported previously that PBMC sensitivity to prednisolone in-vitro was closely related to the clinical efficacy of prednisolone in renal transplant recipients (Hirano et al 1994). Individual differences in PBMC response to prednisolone were observed in our previous studies (Kang et al 1991; Hirano et al 1994, 1997b; Horigome et al 1999) as well as in this study. Furthermore, the data shown in Table 1 indicated that prednisolone sodium succinate IC50s also deviated largely between subjects. On the other hand, the amounts of prednisolone released into the culture medium from prednisolone sodium succinate in the presence of PBMCs from six donors were not significantly different (Table 1). These results suggested that the differences in prednisolone sodium succinate IC50s between subjects were not due to the individual differences of the amount of free prednisolone released into the PBMC culture medium, but may be due to individual variations in intrinsic PBMC sensitivity to prednisolone. The molecular mechanism of the large deviation in the IC50 values of prednisolone sodium succinate is unclear. However, we reported previously that the decreases in PBMC sensitivity to prednisolone in healthy subjects correlates with the decrease in the number of glucocorticoid receptors (Hirano et al 1997a).

Our observations suggest that although prednisolone liberated from prednisolone sodium succinate exhibits immunosuppressive effects in-vitro, its potency is several fold weaker than that of prednisolone if the prodrug is not efficiently hydrolysed in the body. These results raised the possibility that the clinical efficacy of intravenously administered prednisolone sodium succinate may be delayed or attenuated in patients with impaired sodium succinate ester hydrolysis activity, such as those with liver diseases.

References

Hirano, T., Oka, K., Takeuchi, H., Sakurai, E., Matsuno, N., Tamaki, T., Kozaki, M. (1994) Clinical significance of glucocorticoid pharmacodynamics assessed by anti-lymphocyte action in kidney transplantation: marked difference between prednisolone and methylprednisolone. *Transplantation* **57**: 1341–1348

Hirano, T., Abe, K., Gotoh, M., Oka, K. (1995) Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. *Br. J. Cancer* **72**: 1380–1388

Hirano, T., Horigome, A., Oka, K., Takeuchi, H., Yoshida, M., Sakurai, E., Kozaki, K., Matsuno, N., Nagao, T., Kozaki, M. (1997a) Glucocorticoid-resistance in peripheral-blood lymphocytes does not correlate with number or affinity of glucocorticoid-receptors in chronic renal failure patients. *Immunopharmacology* **36**: 57–67

Hirano, T., Oka, K., Takeuchi, H., Kozaki, K., Matsuno, N., Nagao, T., Kozaki, M., Ichikawa, M., Yoshida, M., Umezawa, Y., Hirata, M., Oh-i, T., Koga, M. (1997b) Immunosuppressant pharmacodymamics on lymphocytes from healthy subjects and patients with chronic renal failure, nephrosis and psoriasis: possible implications for individual therapeutic efficacy. *Clin. Pharmacol. Ther.* **62**: 652–664

Horigome, A., Hirano, T., Oka, K., Takeuchi, H., Sakurai, E., Kozaki, K., Matsuno, N., Nagao, T., Kozaki, M. (1999) Lymphocyte-sensitivity to glucocorticoid correlates with the sensitivity to cyclosporine A and tacrolimus in chronic renal failure patients. *Br. J. Clin. Pharmacol.* **48**: 573–578

Kang, X. X., Hirano, T., Oka, K., Sakurai, E., Tamaki, T., Kozaki, M. (1991) Role of altered prednisolone-specific lymphocyte sensitivity in chronic renal failure as a pharmacodynamic marker of acute allograft rejection after kidney transplantation. *Eur. J. Clin. Pharmacol.* **41**: 417–424

Margaretha, L. P., Bengt, J., Brigitta, A. N., Hans, O. S., Kristin, H., Ronald, W. P. (1994) Non-specific steroidal esterase activity and distribution in human and other mammalian tissues. *Clin. Chim. Acta* **224**: 9–20

Tamm, J. K., Voigt, D. (1960) Experimentelle und klinische Untersuchungen mit synthetischen wasserloslichen Steroidkonjugaten bein Menschen. *Acta Endocrinologica* (*Copenhagen*) **54** (Suppl.): 5–77