Pharmacokinetic and Pharmacoimmunodynamic Interactions Between Prednisolone and Sirolimus in Rabbits

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Purpose. To assess pharmacokinetic and pharmacoimmunodynamic interactions between prednisolone (Pred, 1 mg/kg) and sirolimus (Sir, 0.25 mg/kg) in rabbits.

Methods. After intravenous administration, plasma concentrations of Pred and corticosterone, and Sir blood concentrations were followed for 24 hours along with blood granulocyte and T-helper cell counts. Ex vivo and in vitro whole blood lymphocyte proliferation marked lymphocyte reactivity.

Results. Pred terminal half-life and clearance were 1.1 hr and 0.72 l/hr/kg with no difference after Sir. Sir values were 13 hr and 0.16 l/hr/kg and Pred produced no changes. Corticosterone production (0–12hr) was suppressed by 55% after Pred alone or combined, while Sir did not cause adrenal suppression. Blood T-helper cells and granulocytes displayed circadian rhythms after placebo. Over 12 hr, T-helper cell counts were decreased by Pred (40%) and Sir (19%) while granulocyte numbers increased by 56% and 23%. After coadministration, cell numbers were similar to Pred alone. Pred and Sir decreased lymphocyte reactivity by 41% and 56% over 24 hr and their combination reached 85% inhibition with additive interaction. In vitro studies showed antagonistic or synergistic interactions depending on drug concentration ratios.

Conclusions. At therapeutic concentrations, Sir and Pred do not significantly interact pharmacokinetically and have additive pharmacoimmunodynamics. Thus, the therapeutic application of this combinaison is promising.

KEY WORDS: prednisolone; sirolimus; immunosuppressant; interaction; pharmacokinetics; pharmacodynamics; rabbit.

INTRODUCTION

Sirolimus is a new immunosuppressive compound under clinical investigation and used in combination with cyclosporine, corticosteroids or azathioprine in organ transplantation (1). Sirolimus acts through a unique mechanism by blocking cytokine (eg., IL-2) transductional signals (2) as cyclosporine and glucocorticosteroids primarily decrease IL-2 production (3). Owing to these different mechanisms, the combined effects of these drugs were mainly synergistic as demonstrated in vitro and in vivo (1,4). However, the prednisolone/sirolimus combination was only extensively evaluated in vitro (4).

Additional pharmacodynamic interactions may occur in vivo between sirolimus and prednisolone as prednisolone produces adrenal suppression and alters lymphocyte trafficking (5). In patients, sirolimus coadministration with prednisolone did not further suppress adrenal secretion but cell trafficking was not studied (6). Moreover, these drugs may interact in their pharmacokinetics as they are metabolized by CYP3A4 enzymes (7,8). Tacrolimus, a sirolimus structurally related immunosuppressive compound, inhibits CYP3A substrates in human hepatic microsomes (9). Two weeks of concomitant oral administration resulted in sirolimus decreasing prednisolone apparent clearance by 27% in renal transplant patients (6).

The rabbit was chosen as an animal model as it functions better than rat for studying prednisolone and tacrolimus pharmacokinetics (10,11) and sirolimus whole blood degradation half-life was closer to humans in rabbits than in rats (12). Moreover, rats are an imperfect model for characterizing cell trafficking, in contrast to rabbits (13,14).

The present studies were conducted to assess the pharmacokinetic and pharmacodynamic interactions between prednisolone and sirolimus after intravenous administration. Several markers were examined for possible drug effects including adrenal suppression, alteration of granulocyte and T-helper lymphocyte trafficking, and inhibition of lymphocyte responsiveness. In addition, the nature of prednisolone and sirolimus interaction in inhibiting lymphocyte proliferation was further explored in vitro.

MATERIALS AND METHODS

Animals

New Zealand rabbits (4 males and 2 females) weighing 3.0 to 4.2 kg were purchased from Becken Farm (Sanborn, NY), and housed in a 12 h light/dark cycle and a constant temperature (22°C). Rabbits received 125 g of chow per day (PMI Feeds Inc., St Louis, MO) and had free access to water. Animals were acclimatized for 10 days prior to experiments. On study days, rabbits were placed in metal restrainers at 7 AM and a cannula (20 G, 1 inch long) was inserted into the ear artery. A temporary dosing catheter was placed in the vein of the other ear. After the 12 hr sample, cannulas were removed and rabbits were returned to their cages until the 24 hr time point. The project adhered to the principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

Compounds

Glucocorticosteroids were purchased from Sigma (St. Louis, MO). Sirolimus and ^{14}C-sirolimus were gifts from Wyeth-Ayerst (Pearl River, NJ). Sirolimus was formulated as a 4 mg/ml solution containing N-N-dimethylacetamide, Tween 80, and polyethylene glycol 400 (2:1:7). Prednisolone hemisuccinate was dissolved in saline at an equivalent prednisolone concentration of 22.4 mg/ml. The placebo solution was a 1:1 mixture of saline and sirolimus vehicle.

Experimental Design

The experiment was designed as a 4-way cross-over study with treatments of: prednisolone (1 mg/kg), sirolimus (0.15

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mg/kg), prednisolone and sirolimus in combination (same doses), and placebo. The four phases were randomized using a latin square design and were separated by 2-week washout periods. At 3 weeks prior to the first treatment, 15 ml of blood were taken from each rabbit for use in in vitro studies.

Doses were administered at 8 AM over 1 min and blood samples were drawn at −1, 5, 10, 20, 30, 45, 60 and 90 min, and at 2, 4, 6, 8, 12, and 24 hr. Samples were divided with 0.5 to 1 ml used for sirolimus whole blood determination, 0.5 to 1 ml centrifuged for plasma steroid determination, and 250 μl used for whole blood lymphocyte proliferation (100 μl), leukocyte count (20 μl) and T-helper cell and granulocyte determination (50 μl). Pharmacokinetic samples were immediately frozen at −70°C for later analysis.

Analytical Methods

Prednisolone, prednisone and corticosterone concentrations were measured by a validated high-performance liquid chromatographic assay (15) and was adapted by using rabbit stripped plasma (14). The assay was linear from 5 to 100 ng (corticosterone), 200 ng (prednisone) and 500 ng (prednisolone) for 0.5 ml of plasma. The inter and intraday coefficients of variation were below 8.0% for all steroids tested. Sirolimus concentrations were determined by a validated high-performance liquid chromatographic method (16). The assay was linear from 2.5 to 200 ng for 1.0 ml of whole blood. The inter and intraday coefficients of variation were below 9.4%. There was no interference between steroids and sirolimus.

In Vitro Sirolimus Whole Blood: Plasma Distribution

Rabbit whole blood (1.0 ml) was placed in glass tubes and spiked with a constant quantity of ^{14}C-sirolimus and varying concentrations of sirolimus (5 to 1000 ng/ml). After 15-min incubation at 37°C, 0.1 ml was withdrawn for whole blood radioactivity and plasma was obtained by centrifugation. Blood samples were treated with Soluene-350 (Packard Instruments, Meriden, CT) and hydrogen peroxide. All samples were corrected for quenching.

Pharmacodynamic Measurements

Leukocyte count was performed using an automated Coulter counter (Coulter Electronics Inc., Hialeah, FL). After treating blood samples (50 μl) for 10 min with mouse IgG (Sigma), cells were reacted with mouse anti-CD4 monoclonal IgG_{2a} antibody conjugated with fluorescein isothiocyanate (Serotec, Oxford, England) for 15 min. After proper lysing of red blood cells and washing, samples were analyzed on a FACScan flow cytometer (Becton-Dickinson, Montain View, CA).

The in vitro whole blood lymphocyte proliferation method published by Ferron and Jusko (4) was applied, without modification, for the ex vivo and in vitro lymphocyte proliferation measurements. All samples were plated in quintuplets (ex vivo) or triplicates (in vitro).

Pharmacokinetic Analysis

Prednisolone and sirolimus concentration-time profiles exhibited biexponential decline. They were individually fitted to a two-compartment model in terms of volumes (central volume: Vc; peripheral volume: Vp) and clearances (elimination clearance: CL; distribution clearance: CLd). The differential equations were:

$$\frac{dAc}{dt} = k_0 - (CL + CLd) \cdot \frac{Ac}{Vc} + CLd \cdot \frac{Ap}{Vc} \quad (1)$$

$$\frac{dAp}{dt} = CLd \cdot \frac{Ac}{Vp} - CLd \cdot \frac{Ap}{Vp}$$

where k_0 is the infusion rate and set to zero after 1 min. The volume of distribution at steady-state (Vss) was calculated as Vc + Vp and the mean residence time as Vss/CL. The whole blood:plasma distribution data were fitted to:

$$C_{WB} = C_P + \text{Hematocrit} \cdot \frac{B_{max} \cdot C_P}{K_D + C_P} \quad (2)$$

where C_{WB} is whole blood concentration, C_P is plasma concentration, B_{max} is the binding capacity, and K_D is the binding affinity constant.

Pharmacodynamic Analysis

Granulocyte Count, T-helper Cell Count, and Corticosterone

The area under effect curves from 0 to 12 or 24 hr were computed using splines; values obtained for placebo treatments were used as baseline to calculate the area suppression ratio ($AUEC_{SR}$):

$$AUEC_{SR,\,Drug} = \frac{AUEC^{t}_{0_{Drug}}}{AUEC^{t}_{0_{Placebo}}} \quad (3)$$

Ex Vivo *Lymphocyte Proliferation*

Observed effects were expressed as percent of predose values and $AUEC_{SR}$ values were calculated. Three additional parameters were obtained from direct inspection of the data: the percentage of inhibition at 5 min (I_{5min}), the maximum inhibition value (I_{max}) and the time when I_{max} occurs (t_{max}).

In Vivo *Interaction Analysis*

Assuming additive interaction, the effect of the combination (17) was predicted as:

$$\text{Predicted } AUEC_{SR,Pred+Sir} = AUEC_{SR,Pred} \cdot AUEC_{SR,Sir} \quad (4)$$

These values were statistically compared to the observed values. Any differences indicate either synergism (underprediction of combined effect) or antagonism (overprediction).

In Vitro *Interaction Analysis*

Data were expressed as a percentage of control values and analyzed with isobols and the Universal Response Surface Approach which provides an interaction parameter α (4,18). If α equals zero, the interaction is additive and a positive α indicates synergism.

Statistical Analysis

The pharmacokinetic/pharmacodynamic analysis was performed using the ADAPT-II software (Biomedical Simulations

Resource, Los Angeles, CA) applying the general least-squares procedure. Errors between predicted and observed values were assumed to be normally distributed, and the residual variance was defined as $\sigma_1^2 \cdot Y_i^{\sigma 2}$ where σ_1 and σ_2 are the variance parameters and Y_i is the prediction at time t_i.

Paired t tests were used to compare prednisolone or sirolimus pharmacokinetic parameter values after administration of compounds alone or in combination. The pharmacodynamic differences observed between treatments were assessed by variance analysis using the SAS software (SAS Institute, Cary, NC) with the placebo phase as reference. The significance level of $p < 0.05$ was chosen.

RESULTS

Pharmacokinetics

Prednisolone, prednisone and sirolimus concentration-time profiles are displayed in Figure 1 for one representative rabbit after administration of prednisolone and sirolimus alone or jointly. Drug concentrations followed biexponential declines with a shallower profile for sirolimus. Prednisone, prednisolone reversible inactive metabolite, was detected 5 min and followed for only 2 hours after prednisolone administration. In 4 rabbits, slightly higher prednisolone concentrations were obtained with sirolimus conadministration. Pharmacokinetic parameter values are summarized in Table I for all rabbits as no gender differences were detected.

Prednisolone was quickly metabolized into prednisone and was rapidly distributed and cleared from rabbits as the MRT

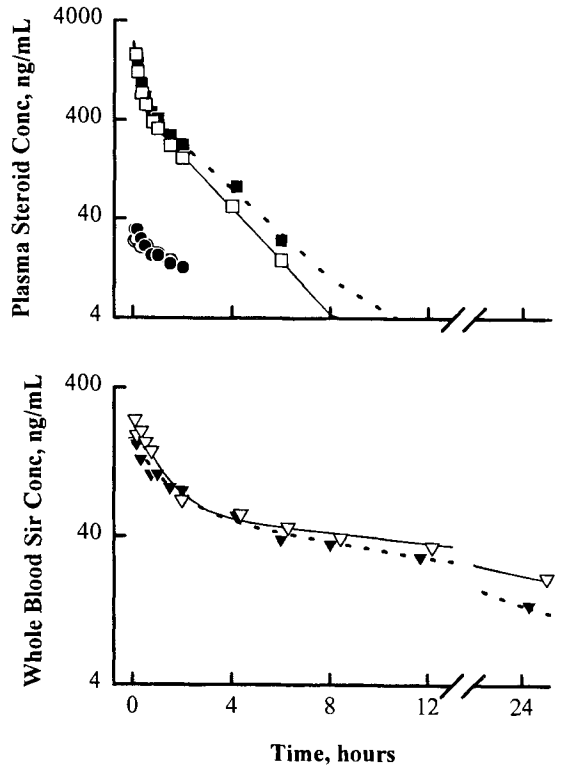

Fig. 1. Prednisolone (squares), prednisone (circles) and sirolimus (triangles) concentration-time profile for one rabbit after administration of prednisolone or sirolimus alone (open symbols) or together (solid symbols). Curves show fitted values.

was 1.29 hr. Prednisolone distributes into the extravascular space with a Vss (0.93 l/kg). Prednisolone has a low hepatic extraction ratio of 0.16 as its total plasma clearance was 0.72 l/hr/kg whereas liver plasma flow is 4.5 l/hr/kg (19).

Sirolimus has a long elimination half-life (13.4 hr) in relation to a low whole blood clearance (0.16 l/hr/kg) and a large Vss (2.88 l/kg) and its extraction ratio was extremely low (0.02). Its in vitro distribution into red blood cells is non-linear with whole blood : plasma ratios ranging from 4.5 to 1.8 (Fig. 2). This was characterized by a B_{max} of 1306 ng/ml and K_D of 180 ng/ml.

No significant pharmacokinetic interaction was found. However, prednisolone elimination half-life was slightly higher after sirolimus coadministration (ratio = 1.15), in relation to a lower prednisolone clearance (ratio = 0.92).

Pharmacodynamics

All pharmacodynamic parameter values are summarized in Table II.

Corticosterone

After placebo, corticosterone concentrations fluctuated with higher values observed prior to dosing. These were not significantly altered by sirolimus (Fig. 3). Prednisolone produced marked adrenal suppression no further modified by sirolimus coadministration as indicated by similar $AUEC_{SR}$ values.

T-helper Lymphocytes

After placebo treatment, blood T-helper cells followed a circadian rhythm with higher cell counts observed at 0 to 1 hour after dosing and at 24 hr; lower counts were measured at 4 to 8 hours (Fig. 4). Lymphocyte trafficking was slightly altered by sirolimus and significantly inhibited by prednisolone. Combination $AUEC_{SR}$ values were comparable to those with prednisolone alone.

Granulocytes

Baseline circulating granulocytes showed fluctuations possibly related to a circadian rhythm with lower cell counts observed prior to dosing and at 24 hr (Fig. 4). Granulocyte trafficking was moderatly altered by sirolimus and, to a greater extent, by prednisolone. Sirolimus did not further modify the effect of prednisolone.

Ex Vivo *Lymphocyte Proliferation*

Some fluctuations were found over time after placebo and, on average, the lowest proliferative response was observed at 4.3 hr and was 51% of the predose level (Fig. 5). Both prednisolone and sirolimus significantly inhibited lymphocyte reactivity and combination values were reasonably predicted by Eq. 4. After prednisolone dosing, the initial 59% inhibition was further increased to a maximum of 91% at 2 to 4 hr.

In Vitro *Lymphocyte Proliferation*

Prednisolone was less efficient and potent than sirolimus in inhibiting lymphocyte proliferation and was 30-times more potent in females. γ values were lower for sirolimus, in relation

Table I. Prednisolone and Sirolimus Pharmacokinetic Parameters After Single and Joint Administration (Mean ± SD)

	CL[a] (l/hr/kg)	CLd (l/hr/kg)	Vc (l/kg)	Vss (l/kg)	$t_{(1/2)\beta}$ (hr)	MRT (hr)
Prednisolone						
alone	0.722 ± 0.093	0.990 ± 0.37	0.406 ± 0.098	0.933 ± 0.13	1.14 ± 0.05	1.29 ± 0.07
with sirolimus	0.660 ± 0.13	0.771 ± 0.26	0.393 ± 0.058	0.914 ± 0.069	1.33 ± 0.25	1.42 ± 0.20
ratio joint/alone	0.921 ± 0.22	0.693 ± 0.84	0.961 ± 0.19	0.995 ± 0.20	1.15 ± 0.17	1.07 ± 0.11
Sirolimus						
alone	0.159 ± 0.046	0.858 ± 0.39	1.03 ± 0.32	2.88 ± 0.87	13.4 ± 4.2	19.2 ± 6.9
with prednisolone	0.166 ± 0.046	0.896 ± 0.58	1.00 ± 0.24	2.37 ± 0.75	11.5 ± 3.8	14.9 ± 5.6
ratio joint/alone	1.07 ± 0.20	0.948 ± 0.46	1.04 ± 0.17	0.861 ± 0.14	0.890 ± 0.14	0.776 ± 0.28

[a] CL, elimination clearance; CLd, distribution clearance; Vc, central volume of distribution; Vp, peripheral volume of distribution; $t_{1/2\beta}$, terminal half-life; MRT, mean residence time.

to greater cell heterogeneity as it acts later in the cell cycle. The nature of their combination was mainly synergistic as reflected by the mean α value of 146. More accurately, synergism was found at low to medium prednisolone/sirolimus concentration ratios and antagonism detected at high ratios, as demonstrated by the isobols (Fig. 6).

DISCUSSION

Pharmacokinetics

After iv dosing, prednisolone was rapidly cleared and partly reversibly metabolized into prednisone. Prednisone concentrations were 10 to 100-times lower than prednisolone. This was in agreement with Rocci and Jusko (10) who found an AUC ratio of prednisolone : prednisone ranging from 13 to 36 after iv administration of 2.5 mg/kg of prednisolone succinate to rabbits. The total plasma clearance of prednisolone was 0.72 l/hr/kg, similar to literature values (0.67 l/hr/kg) (10). However, our MRT was slightly longer (1.29 vs. 0.91 hr) in relation to a larger Vss obtained presently (0.93 vs. 0.61 l/kg). This difference may be related to the prednisolone salt used, as pharmacokinetic differences were observed between prednisolone phosphate and tetrahydrophthalate in humans (20).

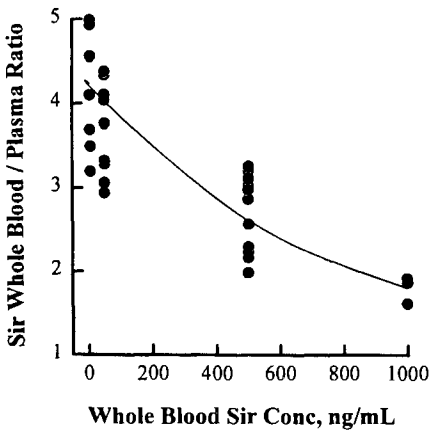

Fig. 2. In vitro whole blood: plasma ratio as a function of sirolimus whole blood concentrations. Data were obtained in different rabbits. Line shows fitting to Eq. 2.

Sirolimus pharmacokinetics differs from prednisolone by its long elimination half-life (13.4 hr) in relation to a low whole blood clearance (0.16 l/hr/kg) and a large Vss (2.72 l/kg). These values are in agreement with Yatscoff *et al.* (21). Sirolimus is extensively distributed into red blood cells in a concentration-dependent fashion as in vitro whole blood : plasma ratios ranged from 4.5 to 1.8 at concentrations of 5 to 1000 ng/ml (Fig. 2). Sirolimus binding to the ubiquitous FKBP12 cytoplasmic protein is responsible for extensive red blood cell distribution (22) and was characterized by a B_{max} of 1.43 μM and K_D of 0.197 μM. Tacrolimus also displays concentration-dependent erythrocyte binding in rabbits and humans (11,23). In rabbits, its whole blood : plasma ratio was around 9 for low plasma concentrations and averaged 1.7 when concentrations were higher than 30 nM. The B_{max} for tacrolimus was 5-times lower than sirolimus (0.29 μM) and the K_D was 15.8 nM (11). The doubled whole blood : plasma ratio for sirolimus was reflected by a one-half lower clearance (0.159 vs. 0.312 l/hr/kg) as erythrocytes protect these compounds from hepatic extraction (11,23). In contrast to tacrolimus, the slight non-linear erythrocyte binding of sirolimus may not be a critical factor in disposition as therapeutic concentrations are about 50 ng/ml (24). In addition, as sirolimus has a 62 hr degradation half-life in rabbit blood, instability may account for 7 to 20% of its total clearance in rabbits (12).

After coadministration with prednisolone, no alteration of sirolimus pharmacokinetics was detected. However, prednisolone elimination may be slightly affected with an 8% decrease in total plasma clearance resulting in an increased elimination half-life. Rabbit drug exposures (AUC) were similar to those of humans receiving 15 mg prednisolone and 5 mg sirolimus oral doses (6). Under these conditions, a modest reduction of prednisolone elimination after sirolimus coadministration was observed in humans (6). Sirolimus is extensively metabolized by CYP3A4 enzymes, a metabolic pathway partly responsible (circa 20%) for metabolism of prednisolone (7,8). Tacrolimus was found to significantly inhibit CYP3A metabolism in human hepatic microsomes (9). Apparently such inhibition is modest in the case of sirolimus and prednisolone in rabbits and humans.

Pharmacodynamics

Corticosterone is the predominant endogenous corticosteroid in rabbits (14) and its levels fluctuate over the day with a

Table II. Prednisolone and Sirolimus Pharmacodynamic Parameters After Single and Joint Administration (Mean Treatment to Baseline Ratio ± SD) or in Whole Blood Lymphocyte Proliferation Assay (Mean ± SD)

	Placebo	Prednisolone	Sirolimus	Combination
T-helper Lymphocytes				
$AUEC_{SR}$ (0–12 hr)	1.00	$0.600^{a,b}$ ± 0.21	0.813^{b} ± 0.14	0.679^{a} ± 0.27
$AUEC_{SR}$ (0–24 hr)	1.00	0.661^{a} ± 0.20	0.843^{b} ± 0.37	0.688^{a} ± 0.23
t_{max}, hr	5.6 ± 3.2	4.4 ± 2.9	3.9 ± 3.5	$8.9^{a,b}$ ± 2.5
Granulocytes				
$AUEC_{SR}$ (0–12 hr)	1.00	1.56^{a} ± 0.40	1.23^{b} ± 0.18	1.50^{a} ± 0.16
$AUEC_{SR}$ (0–24 hr)	1.00	1.35^{a} ± 0.16	1.20^{a} ± 0.19	1.30^{a} ± 0.09
Corticosterone				
$AUEC_{SR}$ (0–12 hr)	1.00	0.452^{a} ± 0.24	1.16^{b} ± 0.59	0.468^{a} ± 0.31
$AUEC_{SR}$ (0–24 hr)	1.00	0.559 ± 0.32	1.08^{b} ± 0.34	0.724 ± 0.50
Ex Vivo WBLP				
$AUEC_{SR}$ (0–12 hr)	1.00	$0.422^{a,b}$ ± 0.12	$0.277^{a,b}$ ± 0.05	$0.117^{a,b}$ ± 0.09
$AUEC_{SR}$ (0–24 hr)	1.00	$0.591^{a,b}$ ± 0.29	$0.443^{a,b}$ ± 0.18	$0.147^{a,b}$ ± 0.13
I_{5min}, %	19.3 ± 29	$58.8^{a,b}$ ± 10.7	89.3^{a} ± 4.9	93.2^{a} ± 5.1
I_{max}, %	48.9 ± 16	90.7 ± 3.7	93.2 ± 5.7	97.4^{b} ± 1.5
Time of I_{max}, hr	4.3 ± 4.7	2.3 ± 1.3	0.30^{b} ± 0.10	4.0 ± 1.3
In Vitro WBLP				
I_{max}, %		61^{b} ± 24	99 ± 1	
$IC_{50,\,Male}$, nM		339^{b} ± 188	6.38 ± 1.45	
$IC_{50,Female}$, nM		10.9^{c} ± 4.4	2.58 ± 0.11	
γ		2.19 ± 1.7	0.90 ± 0.2	
α				146 ± 149

[a] Statistically different from placebo.
[b] Statistically different from other drug treatments.
[c] Statistically different between gender.

maximum observed in the early morning. Prednisolone produced adrenal suppression in rabbits as found in humans (5), but similar experiments with methylprednisolone failed to demonstrate such an effect (14). Rabbits may differ from humans in sensitivity to glucocorticoid effects on corticosterone secretion. As sirolimus did not affect either adrenal function or prednisolone elimination, no further suppression was noted with the drug combination, confirming observations made in renal transplant patients (6).

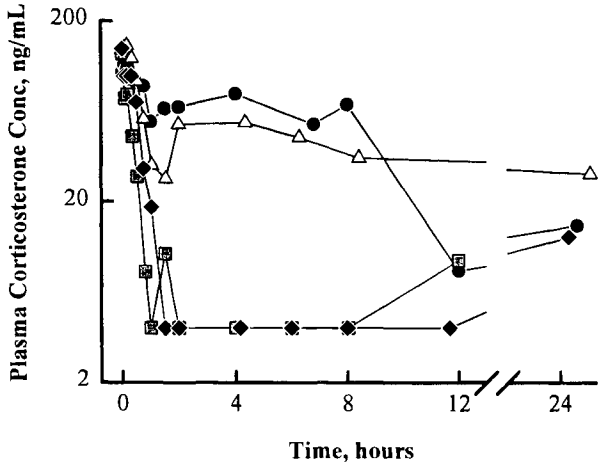

Fig. 3. Plasma corticosterone concentrations in one rabbit after administration of placebo, prednisolone alone, sirolimus alone, and prednisolone/sirolimus jointly (● = placebo, ■ = prednisolone, Δ = sirolimus, ◆ = combination). Lines join observed data.

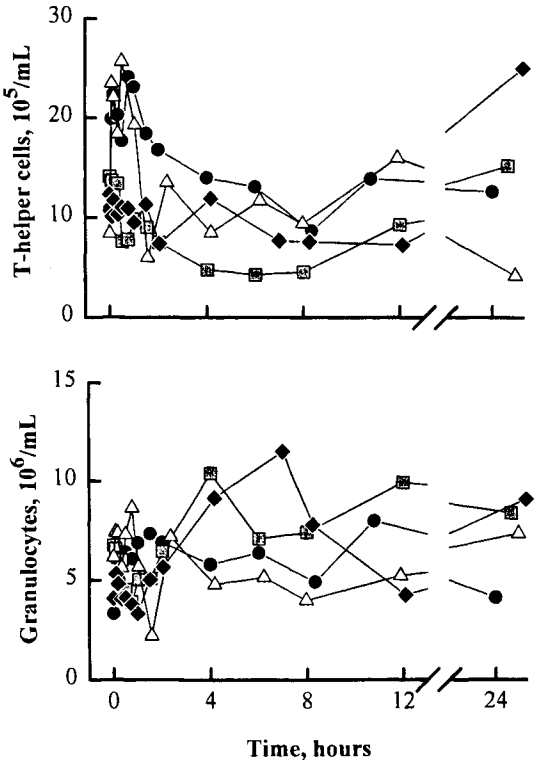

Fig. 4. Time profiles of T-helper lymphocytes and granulocytes in whole blood in one rabbit for the four treatments (● = placebo, ■ = prednisolone, Δ = sirolimus, ◆ = combination). Lines link observed data.

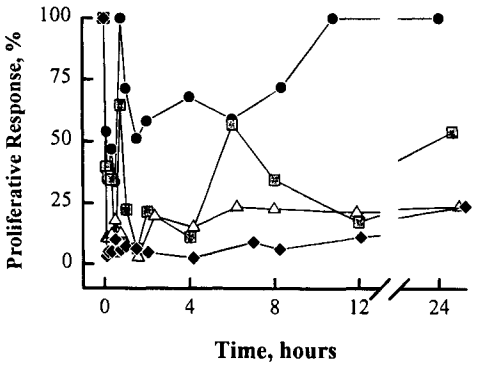

Fig. 5. Ex vivo whole blood lymphocyte proliferative response as a function of time in one rabbit after the four treatments (● = placebo, ■ = prednisolone, Δ = sirolimus, ◆ = combination).

Immunosuppressive and antiinflammatory activities of glucocorticoids are partly related to their redirection of leukocyte trafficking away from secondary lymphoid organs and inflamed sites. In humans, circulating T-helper lymphocytes display a circadian rhythm inversely related to cortisol levels (25). Their numbers decrease shortly after steroid administration, reaching a maximum change several hours later. T-helper lymphocytes display a circadian rhythm in rabbits (14), but no correlation with corticosterone was evident (Fig. 3). After giving prednisolone alone or in combination, similar inhibition of T-helper cell trafficking was observed, as sirolimus had an insignificant effect. In addition to steroid alteration of lymphocyte homing, increased numbers of circulating granulocytes were found in humans. In rabbits, sirolimus had an insignificant effect (23%) compared to prednisolone (56%) or its combination with sirolimus (50%). Therefore, effects of both compounds were additive in altering leukocyte trafficking.

Determination of lymphocyte responsiveness can be obtained ex vivo by performing mitogen-stimulated whole blood lymphocyte proliferation and this method requires very small blood volumes allowing serial sampling (4). This assay reflects the number of lymphocytes present in the blood sample as well as their capability to respond to an immunologic challenge. As T-helper lymphocytes displayed a circadian rhythm, ex vivo proliferative responses fluctuated with time and 51% of the initial 8 AM response was obtained 4.3 hr after placebo administration, in concordance with lower T-helper cell numbers (Fig. 5). Interestingly, maximum inhibition of the proliferative response was not immediately observed after prednisolone, but 2 to 4 hr later, when drug concentrations were below the in vitro IC_{50}. The initial 59% inhibition was confirmed in vitro. Similar values were reported for methylprednisolone (14), categorizing rabbits as a resistant species to steroid direct immunosuppressive action. The greater immunosuppression observed at 2 to 4 hr was in relation to prednisolone alteration of lymphocyte trafficking. After sirolimus, the maximum inhibition was immediately observed and the presence of prednisolone increased the intensity and duration of maximum immunosuppression. From the ex vivo whole blood lymphocyte proliferation assay, it was concluded that the prednisolone/sirolimus combination was additive.

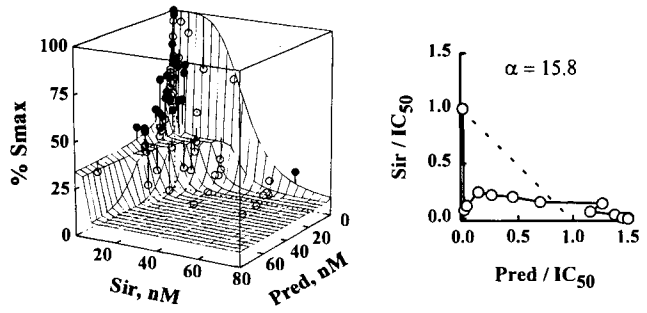

Fig. 6. In vitro whole blood lymphocyte proliferation inhibition as a function of prednisolone and sirolimus concentrations in one rabbit. Data were analyzed with the Universal Response Surface Approach (left panel) yielding the interaction parameter α. Solid symbols are above the surface indicating enhanced effects and open circles are below the surface reflecting reduced effects. The synergistic individual α value is displayed with the isobologram (right panel). Diagonal line represents additivity.

The nature of prednisolone and sirolimus interaction was further studied in vitro using the whole blood lymphocyte proliferation assay. With a 61% maximum inhibition, rabbit lymphocytes were only partially sensitive to prednisolone action. Females were more sensitive than males, confirming results obtained in humans (5). However, no gender difference was evident in vivo. In vitro, prednisolone was less potent than sirolimus in inhibiting lymphocyte proliferation in male rabbits but similar potencies were found in females. The prednisolone/sirolimus interaction was synergistic on average as indicated by positive alpha values but, after studying the isobolograms, the interaction was characterized as biphasic (Fig. 6). The nature of this interaction cannot be easily explained as these drugs act through intricate mechanisms on lymphocyte activation and proliferation (4). Corticosteroids decrease cytokine and adhesion molecule gene transcription after binding to their cytoplasmic and nuclear receptors (3). Sirolimus blocks transductional signals produced by fixation of cytokines or growth factors to their membrane receptors (1,2). Moreover, sirolimus binds to the cytoplasmic glucocorticoid receptor complex (26), potentially altering its functionality, and partially blocks P-glycoprotein function, thus possibly decreasing steroid efflux from cells (26). Steroids further alter the immunologic system in vivo making uncertain the extrapolation of in vitro results to in vivo settings as confirmed with the present study. Indeed, as prednisolone concentrations were below those of sirolimus by 4 hr after administration, strong synergism should have been observed ex vivo.

In conclusion, after iv administration of pharmacologic doses of prednisolone and sirolimus to rabbits, no significant pharmacokinetic interactions were found. Sirolimus did not significantly modify corticosterone levels or circulating granulocyte and T-helper lymphocyte numbers. However, prednisolone altered all these parameters to a similar extent alone or in combination with sirolimus. Ex vivo lymphocyte proliferation was successfully applied for the first time in rabbits and was demonstrated to be of value. Prednisolone and sirolimus interactions were biphasic in vitro and mainly additive in vivo at the pharmacoimmunodynamic level. These results are promising indications for the therapeutic application of the prednisolone/sirolimus combination as these agents display nonoverlapping major side effects (27,28,29).

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