



Determination of the effect of calcineurin inhibitors on the rat's immune system after KLH immunisation

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Abstract

The calcineurin inhibitors cyclosporin A (CsA), tacrolimus (FK506) and pimecrolimus (ASM981) are on the market for the oral treatment of psoriasis and atopic dermatitis and topical treatment of atopic dermatitis, respectively. The effect of these treatments on the immune response was investigated in this study after immunisation of rats with keyhole limpet hemocyanin (KLH). Male rats (10 per group) were orally administered pimecrolimus at 10 or 30 mg/kg/day, tacrolimus at 3 mg/kg/day or CsA at 20 mg/kg/day for 4 weeks. Control animals similarly received the vehicle only. The last five animals per group were immunised and challenged with KLH on days 16 and 24, respectively. Eight days after the last injection, the immune function was investigated by detecting KLH-specific antibodies in the serum and by examination of cell infiltration at the site of the KLH-challenge. In addition, a correlation between functional and structural changes was established by quantification of lymphocyte sub-populations in the blood or residing in lymphatic tissue. In KLH-immunised rats, CsA caused complete suppression of the KLH-specific IgM and IgG production, whereas only IgG production was affected by pimecrolimus at 30 mg/kg/day and more so by tacrolimus at 3 mg/kg/day. Immunophenotyping of lymphocyte sub-populations in spleen and lymph node indicated a decrease in T lymphocytes with pimecrolimus at 30 mg/kg/day, tacrolimus and CsA, whereas these changes were marginal for pimecrolimus at 10 mg/kg/day. Immunophenotyping of peripheral white blood cells (WBC) revealed a decrease in the absolute number of T lymphocytes with all three test items. In comparison with non-immunised animals, a slight increase in absolute numbers of T lymphocytes was observed in KLH-immunised animals treated with pimecrolimus at 10 or 30 mg/kg/day. In conclusion, the ability of the immune system to respond to KLH was not affected by pimecrolimus at 10 mg/kg/day whereas a decrease in immune function was noted in the other groups as follows: pimecrolimus (30 mg/kg/day) < tacrolimus (3 mg/kg/day) < CsA (20 mg/kg/day).

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1. Introduction

The calcineurin inhibitors cyclosporin A (CsA), tacrolimus (FK506) and pimecrolimus (ASM981) are on the market for the oral treatment of psoriasis and atopic dermatitis and topical treatment of atopic dermatitis, respectively (Ohtsuki et al., 2003; Leung and Boguniewicz, 2003; Eichenfield and Beck, 2003). CsA

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and tacrolimus are also used in transplantation patients to prevent transplant organ rejection (Takatsuka et al., 2003). Pimecrolimus, although sharing with the other two compounds the same basic mode of action, appears to have lower potential to affect systemic immune reactions as indicated by the results of preclinical studies where the effects of the three compounds were compared in animal models for immunosuppression (Meingassner et al., 1997; Stuetz et al., 2001). Considering their effects on T lymphocytes, concern has been put forward that immunomodulators like calcineurin inhibitors might affect vaccination, especially when they are used in children (Laube et al., 2002).

An increasing awareness of immunotoxic side effects of drugs has triggered the release of a CPMP-EMA guidance document stating that “all new medicinal products should be screened for immunotoxic potential in at least one repeated-dose toxicity study” (European Agency for the Evaluation of Medicinal Products, 2000, CPMP/SWP/1042/99). In a tiered testing approach the antibody response to a T-dependent antigen should be tested. Interpretation of data should be performed by applying an holistic approach, which considers histopathology, haematology and other data types. In the event of a proven drug-mediated effect on the immune response, further studies must be conducted in order to clarify the nature of the immunotoxic effect.

The aim of this study was to assess the extend of immunomodulation elicited by the calcineurin inhibitors pimecrolimus, tacrolimus and CsA in a rodent immune response model. Immunisation was performed with keyhole limpet hemocyanin (KLH), a soluble protein antigen that results in specific antibody production requiring T- and B-lymphocyte collaboration as well as antigen presenting cells (Irwin, 1993).

2. Materials and methods

2.1. Animals treatment

Groups of 10 male Wistar rats were treated for 4-weeks, by oral gavage, with pimecrolimus (ASM981, Novartis Pharma AG, Switzerland) at 10 or 30 mg/kg/day, tacrolimus (Prograf[®] 5 mg capsules, Fujisawa Ltd, Ireland) at 3 mg/kg/day and CsA

(Sandimmun[®] Neoral[®] Drink Solution 100 mg/ml, Novartis Pharma AG) at 20 mg/kg/day using a dosage volume of 5 ml/kg. Control animals similarly received the placebo for pimecrolimus (aqueous solution containing cellulose-HP-603 and lactose). The last five animals per group were immunised (day 16) and challenged (day 24) sub-cutaneously with KLH at 50 µg per animal in a formulation containing the aluminium hydroxide adjuvant (AluGel-S-Suspension, SERVA Electrophoresis GmbH, Germany). The necropsy was performed on days 31 (non-immunised animals, five per group) and 32 (KLH-immunised animals, five per group).

2.2. Blood sampling for immune function testing

Blood samples were taken from the sub-lingual (tongue) vein during inhalation anaesthesia with Isoflurane (Forene[®]; Abbott Laboratories S.A., Switzerland) before necropsy. Samples were taken into EDTA coated tubes for haematology and immunophenotyping of lymphocytes. Serum was separated and frozen pending detection of KLH-specific antibodies.

2.3. Haematology

Total and differential white blood cells (WBC) counts were performed using an ADVIA 120 (Bayer Diagnostics, Switzerland).

2.4. Detection of KLH-specific antibodies in serum

KLH-specific serum antibodies were determined on ELISA plates coated with a 1 µg/ml KLH solution (Sigma, Switzerland) in coating buffer (KPL, Switzerland) for 1 h at room temperature. After blocking with assay diluent (Pharmingen, Switzerland) for 1 h at 37 °C, plates were incubated with serial dilutions of the serum samples, in triplicate, for 2 h at 37 °C. This was followed by incubation (1 h at 37 °C) with an anti rat IgM or IgG detection antibody coupled with biotin (1/3000, Pharmingen) and subsequent incubation (1 h at 37 °C) with a streptavidin horseradish peroxidase complex (1/1000, Pharmingen). After stopping the tetramethylbenzidine (TMB substrate reagent set, Pharmingen) colour reaction with sulphuric acid, the optical density (OD) was measured at 450 nm. Washing steps (wash solution, KPL) were performed after

each incubation. A threshold optical density for the determination of the antibody titre was established using the results from the non-immunised control animals. Values represent the dilution factors (titre) corresponding to this threshold OD and were calculated by linear interpolation from the OD curve of the particular serial dilution. In the event that the complete OD curve of a particular sample was below the threshold value, the titre was defined as a value of "1".

2.5. Immunophenotyping of peripheral lymphocytes

After lysis of erythrocytes (Uti-Lyse, Dako, Switzerland), the white blood cells were centrifuged on glass slides with the Shandon cytocentrifuge (Cytospin 2, Shandon Co., ca 7400002, Switzerland) and stored at approximately -80°C pending evaluation. All antibodies were purchased from Serotec (Switzerland). Cells were stained with either biotin or FITC-conjugated anti-CD4 (W3/25 at 1/75), anti-CD8 (OX-8, 1/75), anti-CD3 (1F4, 1/50) and anti-CD45RA (OX-33, 1/50) antibodies. For the biotin-coupled antibodies, the staining was followed by a second incubation step with streptavidin coupled to Alexa 633 (1/200, Molecular Probes, Switzerland). Analyses were performed by a laser scanning cytometer interfaced to a Compaq computer equipped with the Win-Cyte 3.4 software (Compucyte, Cambridge, MA). For the purpose of this study, the contouring parameter was the propidium iodide (Sigma) fluorescence and the cells expressing CD4, CD8, CD3 and CD45RA were gated on green and far red integral fluorescence. Overlapping nuclei were automatically excluded from the counting by special statistical filters.

Absolute numbers of blood lymphocyte populations were calculated for each animal by multiplying the relative percentages obtained for each cell type by total white blood cell counts.

2.6. Immunophenotyping of axillary lymph node and splenic lymphocytes

The spleen and lymph node samples were placed in PBS buffer (without Mg^{2+} and Ca^{2+}) and stored on ice prior to preparation of single-cell suspensions. For the spleen, erythrocytes were lysed with red blood cell lysing buffer (Sigma). Splenic and axillary lymph node cell suspensions were refrigerated

pending staining with antibodies. All reagents were purchased from Pharmingen. For each sample, 2×10^6 cells were stained with either PerCP-, APC-, PE- or FITC-conjugated anti CD8- (OX-8, 1/200), anti CD3- (1F4, 1/200), anti CD4- (OX-35, 1/200) and anti CD45RA (OX-33, 1/800) antibodies. After staining with antibodies, cells were washed and re-suspended in PBS for flow cytometric analyses, which were performed using a FACSCalibur flow cytometer equipped with Cell Quest software (Becton Dickinson). For the axillary lymph node, only the immunised animals were evaluated.

2.7. Macroscopic and microscopic evaluation

At necropsy, animals were examined macroscopically. Tissue samples from the injection site (challenge) and the mandibular lymph node were examined microscopically after H/E staining.

2.8. Statistical analysis

For haematology, Dunnett's test and Steel's test were used for parametric and non-parametric group comparisons, respectively.

For immunological parameters (i.e. antibody titres and immunophenotyping of the spleen and lymph nodes), all statistical calculations were performed with the software SigmaStat for Windows (Version 2.03) using a one way analysis of variance (Glantz, 1992). A normality test was performed to assure that the samples were drawn from a normal population (P -value to reject = 0.1). The equal variance test was used to display whether or not the data passed or failed the test of the assumption that the samples were drawn from populations with the same variance (P -value to reject = 0.1). In case of significant results of the one-way-ANOVA pairwise comparisons were performed with the Student's–Newman–Keuls test. The confidence interval for the difference of the means was set to 95% ($\alpha = 0.05$).

3. Results

All animals survived the treatment period. There were no clinical signs of toxicity attributable to the treatment with the test items.

3.1. Haematology

A decrease in mean total white blood cell counts was observed in non-immunised animals treated with pimecrolimus at 10 or 30 mg/kg/day (dose-dependent), tacrolimus and CsA, attaining statistical significance for pimecrolimus at 30 mg/kg/day and CsA when compared with controls (Table 1). This was mainly due to a reduction in absolute neutrophil and lymphocyte counts. In KLH-immunised animals, a similar decrease in total white blood cells and absolute neutrophil and lymphocyte counts was found in tacrolimus-treated animals. In animals treated with pimecrolimus, and to a lesser extent with CsA, mean values of total white blood cells, absolute neutrophil and lymphocyte counts indicated a tendency for increased values compared with the non-immunised animals (Table 1).

3.2. KLH-specific antibodies in serum

In general, large inter-individual variations in the antibody titres were observed. Relative KLH-specific

IgM and IgG titres demonstrated successful immunisation except for the CsA-treated rats, which showed titres in the range of the non-immunised controls (Table 2). In the other groups, KLH-specific relative IgM titres were found within the range of the immunised control rats, whereas KLH-specific relative IgG titres were reduced for pimecrolimus at 30 mg/kg/day and, to a greater extent, for tacrolimus at 3 mg/kg/day.

3.3. Immunophenotyping of peripheral lymphocytes

Decreases in the absolute number of T lymphocytes (CD3, CD4 and CD8) were observed for all three test items when compared to control animals (Table 3). The decrease in CD3 lymphocytes was statistically significant in all non-immunised treated groups and in KLH-immunised animals administered tacrolimus or CsA. In comparison with similarly treated non-immunised animals, an increase in absolute T cell numbers was observed in KLH-immunised animals treated with pimecrolimus at 10 or 30 mg/

Table 1

Mean values of absolute numbers of white blood cells, lymphocytes, neutrophils and monocytes in peripheral blood

		Control (cells/ml ($\times 10^6$))	ASM981 (10 mg/kg/day) (cells/ml ($\times 10^6$))	ASM981 (30 mg/kg/day) (cells/ml ($\times 10^6$))	FK506 (3 mg/kg/day) (cells/ml ($\times 10^6$))	CsA (20 mg/kg/day) (cells/ml ($\times 10^6$))
WBC						
KLH-immunised	Mean	7.13	5.99	5.41	4.49	5.34
	S.D.	1.80	2.40	2.16	0.84	1.58
Non-immunised	Mean	7.61	5.85	4.35*	5.18	4.23*
	S.D.	1.61	1.43	0.72	0.87	0.80
Lymphocytes						
KLH-immunised	Mean	5.27	3.63	3.50	2.99	3.47
	S.D.	1.81	0.76	1.63	0.70	1.26
Non-immunised	Mean	5.85	4.57	3.10*	3.74	2.77
	S.D.	1.07	1.43	0.51	0.65	0.31
Neutrophiles						
KLH-immunised	Mean	1.42	1.86	1.35	1.02	1.28
	S.D.	0.20	1.59	0.46	0.13	0.77
Non-immunised	Mean	1.37	0.95	0.87	1.03	0.67
	S.D.	0.84	0.21	0.15	0.23	0.18
Monocytes						
KLH-immunised	Mean	0.23	0.22	0.24	0.24	0.39*
	S.D.	0.04	0.08	0.05	0.07	0.11
Non-immunised	Mean	0.20	0.16	0.18	0.18	0.33
	S.D.	0.08	0.05	0.08	0.06	0.09

ASM981: pimecrolimus, FK506: tacrolimus, CsA: cyclosporin A.

* $P < 0.05$ vs. corresponding control group (immunised or non-immunised).

Table 2
Mean values of KLH-specific Ig titres

		IgM relative titre	IgG relative titre
Control non-immunised	Mean	$1.89 \times 10^{2*}$	1.96^*
	S.D.	1.37×10^2	3.05
Control KLH-immunised	Mean	3.20×10^2	1.04×10^5
	S.D.	5.38×10^2	7.45×10^4
ASM981 KLH-immunised (10 mg/kg/day)	Mean	7.52×10^3	1.09×10^5
	S.D.	7.60×10^3	9.67×10^4
ASM981 KLH-immunised (30 mg/kg/day)	Mean	8.35×10^3	2.64×10^3
	S.D.	9.79×10^3	2.44×10^3
FK506 KLH-immunised (3 mg/kg/day)	Mean	2.99×10^3	7.94×10^2
	S.D.	4.54×10^3	1.16×10^3
CsA KLH-immunised (20 mg/kg/day)	Mean	$1.09 \times 10^{2*}$	1.00^*
	S.D.	5.72×10^1	0.00

Relative titre: dilution of serum samples corresponding to an arbitrary defined threshold optical density. ASM981: picrolimus, FK506: tacrolimus, CsA: cyclosporin A.

* $P < 0.05$, comparison with KLH-immunised controls.

Table 3
Mean values of absolute numbers of CD3-, CD4-, CD8- and CD45RA-positive cells in peripheral blood mononuclear cells

		Control (cells/ml ($\times 10^6$))	ASM981 (10 mg/kg/day) (cells/ml ($\times 10^6$))	ASM981 (30 mg/kg/day) (cells/ml ($\times 10^6$))	FK506 (3 mg/kg/day) (cells/ml ($\times 10^6$))	CsA (20 mg/kg/day) (cells/ml ($\times 10^6$))
CD3						
KLH-immunised	Mean	4.16	3.44	3.34	2.49*	2.80*
	S.D.	0.74	1.00	0.35	0.39	0.96
Non-immunised	Mean	4.30	2.72*	2.49*	2.82*	2.05*
	S.D.	1.15	0.99	0.49	0.72	0.38
CD4						
KLH-immunised	Mean	2.82	2.20	1.97	1.57*	1.85*
	S.D.	0.22	0.48	0.32	0.47	0.87
Non-immunised	Mean	2.82	1.53	1.48*	1.38*	1.51*
	S.D.	0.95	0.49	0.37	0.21	0.32
CD8						
KLH-immunised	Mean	0.82	0.66	0.60	0.60	0.54
	S.D.	0.18	0.18	0.08	0.17	0.25
Non-immunised	Mean	0.92	0.54*	0.54*	0.66	0.41*
	S.D.	0.25	0.19	0.15	0.09	0.09
CD45RA						
KLH-immunised	Mean	2.14	1.81	1.74	1.54	1.03*
	S.D.	0.59	0.32	0.46	0.45	0.32
Non-immunised	Mean	2.05	1.95	1.73	1.62	0.60*
	S.D.	0.41	0.84	0.37	0.47	0.21

ASM981: picrolimus, FK506: tacrolimus, CsA: cyclosporin A.

* $P < 0.05$ vs. corresponding control group (immunised or non-immunised).

Table 4
Mean values of spleen cell surface markers

		Percentage of cells			
		CD3+CD4+	CD3+CD8+	CD3+	CD45RA+
Control KLH-immunised	Mean	28.71 [§]	13.38	39.56	39.86
	S.D.	0.70	1.58	2.12	4.17
Control non-immunised	Mean	25.50	14.80	36.30	44.30
	S.D.	2.40	3.00	4.20	5.70
ASM981 KLH-immunised (10 mg/kg/day)	Mean	25.53 [§]	14.22	36.19 [§]	48.04*
	S.D.	1.93	1.70	2.60	3.33
ASM981 non-immunised (10 mg/kg/day)	Mean	19.90*	12.50	29.30*	50.40
	S.D.	2.30	1.50	1.40	2.40
ASM981 KLH-immunised (30 mg/kg/day)	Mean	22.76 [§]	11.38	31.15* [§]	51.25*
	S.D.	2.23	1.59	1.98	3.40
ASM981 non-immunised (30 mg/kg/day)	Mean	16.00*	9.20	22.70*	56.40*
	S.D.	2.00	1.80	3.60	3.80
FK506 KLH-immunised (3 mg/kg/day)	Mean	20.32* [§]	10.81	28.19* [§]	54.62*
	S.D.	0.89	1.37	2.13	3.41
FK506 non-immunised (3 mg/kg/day)	Mean	16.50*	9.40	23.10*	55.40*
	S.D.	2.10	1.40	2.40	3.80
CsA KLH-immunised (20 mg/kg/day)	Mean	15.76* [§]	10.05	22.99* [§]	48.64*
	S.D.	2.70	3.59	5.73	7.44
CsA non-immunised (20 mg/kg/day)	Mean	12.50*	7.10*	16.60*	47.10
	S.D.	2.30	0.90	2.40	3.70

ASM981: pimecrolimus, FK506: tacrolimus, CsA: cyclosporin A.

* $P < 0.05$ vs. corresponding control group (immunised or non-immunised).

§ $P < 0.05$, comparison immunised vs. non-immunised within the same group.

kg/day. Animals treated with CsA showed also a statistically significant decrease in the absolute number of B cells (CD45RA).

3.4. Immunophenotyping of axillary lymph node and splenic lymphocytes

In the spleen (Table 4), a statistically significant reduction in the relative count of CD3 lymphocytes associated with decreased CD4 values was observed for all test items in the following order of severity: CsA > tacrolimus > pimecrolimus (30 mg/kg/day). A statistically significant reduction was also observed for non-immunised animals administered pimecrolimus at 10 mg/kg/day, but not in similarly treated immunised animals. An increase in the relative count of B cells (CD45RA) in all treated groups was observed. Since an absolute increase of B cells counts was not observed in the periphery, this apparent increase in the relative count is considered not to be relevant.

In the axillary lymph node (Table 5), a slight to moderate, statistically significant reduction of the mean relative count of CD3 lymphocytes associated with decreased CD4 and CD8 values was observed with pimecrolimus at 30 mg/kg/day, tacrolimus and with CsA, without a clear difference in the severity of this finding between the compounds. A slight reduction was also observed with pimecrolimus at 10 mg/kg/day, but without statistical significance. An increase in the relative count of B cells (CD45RA) was observed for all treated groups and, as for the spleen, is considered not to be relevant.

3.5. Macroscopic and microscopic evaluation

At the injection site, minimal to marked granulomatous inflammation, with central caseous necrosis surrounded by fibrosis/granulation tissue, inflammatory cells (mainly lymphocytes and plasma cells) and haemorrhage, was observed in all KLH-immunised

Table 5
Mean values of cell surface markers in the draining axillary lymph node

		Percentage of cells			
		CD3+CD4+	CD3+CD8+	CD3+	CD45RA+
Control KLH-immunised	Mean	51.67	15.50	65.40	28.54
	S.D.	5.65	1.89	4.30	3.37
ASM981 KLH-immunised (10 mg/kg/day)	Mean	42.55	15.30	56.55	33.55
	S.D.	5.66	3.15	7.19	6.97
ASM981 KLH-immunised (30 mg/kg/day)	Mean	38.85	10.34*	48.33*	43.56*
	S.D.	6.05	1.99	6.33	6.02
FK506 KLH-immunised (3 mg/kg/day)	Mean	41.12	11.16*	49.77*	42.17*
	S.D.	10.43	1.80	10.43	10.95
CsA KLH-immunised (20 mg/kg/day)	Mean	43.54	10.31*	51.56*	41.76
	S.D.	11.14	1.51	10.95	10.02

ASM981: pimecrolimus, FK506: tacrolimus, CsA: cyclosporin A.

* $P < 0.05$ vs. corresponding control group (immunised or non-immunised).

groups. The largest lesions with extended necrosis and pronounced granulation tissue reaction were recorded in animals receiving pimecrolimus (both dosages) and its placebo. Animals treated with pimecrolimus at 30 mg/kg/day showed a marked fibrosis and inflammatory cell infiltration, while the weakest fibrous and inflammatory response was noted in the CsA-treated animals.

The mandibular lymph node showed germinal centre development close to the normal level in the pimecrolimus-treated groups immunised with KLH, while the lymph nodes in the tacrolimus- and CsA-treated groups were nearly deprived of germinal centres and partly showed lymphoid atrophy. In non-immunised animals, germinal centre development was similarly reduced with pimecrolimus at 30 mg/kg/day and tacrolimus, and was completely inhibited in CsA-treated animals. In the latter group, this was occasionally associated with lymphoid atrophy of the paracortex.

4. Discussion and conclusion

Oral administration of the calcineurin inhibitors pimecrolimus (10 or 30 mg/kg/day), tacrolimus (3 mg/kg/day) and CsA (20 mg/kg/day) to non-immunised rats, for a period of 4 weeks, resulted in immunological and morphological changes consistent with their

pharmacological activity (Spencer et al., 1997; Henry, 1999; Bornhovd et al., 2001). Immune function testing was assessed by titration of antibodies following immunisation of rats with KLH. In addition, the challenge with KLH (day 24) during the course of this 4-week-study allowed us to check the T- and B-lymphocyte interaction, leading to immunoglobulin class switching, as one of the hallmarks of a secondary immune response.

Successful immunisation of animals was generally confirmed by increased serum titres of KLH-specific IgM and IgG and was associated with granuloma formation (with central caseous necrosis and inflammation) at the injection site. However, IgM and IgG titres of CsA-treated animals were similar to non-immunised controls, indicating complete inhibition of antibody production. KLH-specific IgM titres were similar for immunised controls, pimecrolimus- and tacrolimus-treated animals whereas the IgG titres obtained with pimecrolimus (30 mg/kg/day) and tacrolimus were lower than those of immunised controls. These results indicate that pimecrolimus and tacrolimus did not alter the initial generation of the antibody response in contrast to CsA, but rather affected antibody class switching from IgM to IgG.

In addition to the titration of KLH-specific antibodies and in order to augment the histopathological evaluation, immunophenotyping of lymphocytes in peripheral blood, spleen and axillary lymph nodes was

performed by flow/laser scanning cytometry. Flow cytometric evaluation of immunological parameters are considered beneficial in supplementing histopathology by some authors (ILSI, 1999) and have been successfully used (Burchiel et al., 1997; Vohr, 1995).

A moderate decrease in T lymphocytes in peripheral blood, spleen and the axillary lymph node was observed for pimecrolimus at 30 mg/kg/day, tacrolimus and CsA, whereas these changes were only slight for pimecrolimus at 10 mg/kg/day. This is consistent with the inhibition of antibody formation and a decreased severity of granuloma formation at the injection site and germinal centre development in the mandibular lymph node (tacrolimus < CsA).

In conclusion, the ability of the immune system to respond to KLH was not affected by pimecrolimus at 10 mg/kg/day whereas a decrease in immune function was noted in the other groups as follows: pimecrolimus (30 mg/kg/day) < tacrolimus (3 mg/kg/day) < CsA (20 mg/kg/day).

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