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The anti-arthritic and immunosuppressive effects of cyclosporin A on collagen-induced arthritis in the rhesus monkey

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SUMMARY

The influence of cyclosporin A (CsA) on type II collagen-induced arthritis (CIA) in the rhesus monkey has been investigated. CsA was administered subcutaneously in a dose of 25 mg/kg per day during 9–18 days and additionally 12·5 mg/kg per day for 7 days. At this dosing regime no significant alterations of haematologic parameters were found, indicating that the toxicity of CsA was negligible. Administration of CsA after onset of arthritis had no beneficial effect, but when given between immunization and manifestation of clinical symptoms, CIA could be prevented completely. Moreover, these monkeys became resistant to the disease, because no arthritic activity could be observed upon a booster immunization with type II collagen (CII). The suppression of disease by CsA is reflected in reduced antibody levels to CII.

Keywords collagen arthritis rhesus monkey cyclosporin A

INTRODUCTION

Collagen-induced arthritis (CIA) in rhesus monkeys shares many features with human rheumatoid arthritis [1]. Susceptible rhesus monkeys develop arthritis after a single immunization with bovine type II collagen (B-CII) or rhesus monkey type II collagen [2]. About 20% of the animals appeared to be resistant to the disease, and all shared one particular MHC class I allele (Mamu A26) [3]. So, the absence of the Mamu A26 allele has a great predictive value for susceptibility to the disease. Because of the high disease incidence in animals selected for the absence of Mamu A26, the efficacy and potential toxicity of antirheumatic and immunosuppressive drugs can be evaluated in small panels of animals.

In vitro studies indicated that T cell reactivity to CII is the driving force of CIA in the rhesus monkey [2], but its role *in vivo* remains to be established. Several lines of evidence suggest that the immunosuppressive action of cyclosporin A (CsA) is predominantly limited to T cell-mediated immune responses [4,5] by inhibiting the production of lymphokines [6]. CsA was demonstrated to be effective in rhesus monkeys in transplantation as well as in autoimmune disease [7–9]. In man CsA has been shown effective in different T cell-mediated autoimmune diseases like type I diabetes and rheumatoid arthritis [10,11]. Down-regulation of the anti-CII antibody production by CsA, together with a remission of disease activity, has been demonstrated in a patient with relapsing polychondritis [12].

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In this study the anti-arthritic and immunosuppressive effects of CsA in rhesus monkey CIA were investigated. CsA was administered at different phases during the disease process, namely between immunization and manifestation of clinical arthritis or during clinically active CIA. The results show that CsA suppresses clinical manifestation of CIA and autoantibody production when given before onset of the disease, but not when administered during clinically active CIA.

MATERIALS AND METHODS

Animals

Unrelated male rhesus monkeys (*Maccaca mulatta*) lacking the Mamu A26 allele were selected. They were all born and raised in the primate colony of the institute. The monkeys were observed daily for changes in behaviour, food intake and appearance. When necessary for handling the animals were sedated with 0·1 mg/kg ketamin (10 mg/ml; Chassot & Cie AG, Bern, Switzerland). During the studies the monkeys received standard food pellets (Hope Farms) and drinking water *ad libitum*.

Induction and assessment of arthritis

The rhesus monkeys were immunized with 1·0 mg B-CII extracted from bovine articular cartilage [1]. B-CII was dissolved in 0·1 M acetic acid (2 mg/ml) and emulsified in an equal volume of Freund's complete adjuvant (FCA; Difco Labs, Detroit, MI) by intracutaneous injections on the back. Two different batches of B-CII were used for CIA induction in the two separate experiments described below. Onset of arthritis

Table 1. Effect of cyclosporin A (CsA) on collagen-induced arthritis (CIA) in the rhesus monkeys when given before the disease induction (a) and/or during active disease (b)

Rhesus monkey	Body weight (kg)	CsA (days)	STS (days)	ESR raised at day	CRP raised at day
(a) *CsA administered before disease onset*					
8666	7·8	No	21–38‡	21,28 (90)	14,28 (177)
1JE	8·0	No	24–44‡	24–44 (145)	4–44 (100)
1UR	7·3	No	20–28§	20–42 (105)	0–28 (183)
2BX	6·8	No	28–49¶**	28–44 (60)	14–44 (315)
BB59	6·1	7–32*	Absent	28 (48)	14,28 (183)
BB73	4·5	7–32*	Absent	None	14 (23)
8719	7·2	7–32*	Absent	None	14,28 (9)
8623	5·6	7–32*	Absent	None	14,28 (14)
(b) *CsA administered during clinically active CIA*					
4116	6·0	48–57†	45–86**††	52–61 (18)	48–84 (120)
2774	10·4	48–58†	41–59	49–57 (124)	41–57 (132)

STS, Soft tissue swelling of joints; ESR, erythrocyte sedimentation rate, in parentheses the highest measured value (mm/*l*) during the period mentioned; CRP, C-reactive protein, in parentheses the highest measured value (mg/*l*) during the period mentioned.

* Cyclosporin A dose: days 7–25: 25 mg/kg per day subcutaneously; days 25–32: 12·5 mg/kg per day subcutaneously.

† Cyclosporin A dose: 25 mg/kg per day subcutaneously.

‡ Days 21–42: phenylbutazone 60 mg (3× day intramuscularly) + buprenorfin 0·1 mg (3× day intramuscularly).

§ Days 20–24: phenylbutazone 60 mg (3× day intramuscularly) + buprenorfin 0·1 mg (3× day intramuscularly); days 24–28: buprenorfin 0·1 mg (3× day intramuscularly) + acetylsalicylate 270 mg.

¶ Days 28–35: phenylbutazon 60 mg (3× day intramuscularly) + buprenorfin 0·1 mg (3× day intramuscularly); days 35–42: phenylbutazon 60 mg (3× day intramuscularly) + buprenorfin 0·1 mg (3× day intramuscularly) + acetylsalicylate 270 mg; days 42–44: cortisone 50 mg (3× day intramuscularly).

** Euthanization of the animal due to severe disease.

†† Days 64–78: buprenorfin 0·1 mg (3× day intramuscularly) + dexamethasone 0·25 mg (2× day); days 78–86: buprenorfin 0·1 mg (3× day intramuscularly) + dexamethasone 0·13 mg (2× day).

was assessed by daily observation. Normally, CIA is most manifest by soft tissue swelling (STS) of the small joints of hands and feet, but ankles and wrists can also be affected [1]. Therefore STS of these joints was recorded weekly.

Experimental design

CsA (Sandoz Ltd., Basel, Switzerland) was provided in powder form and dissolved (100 mg/ml) in a mixture of Miglyol and ethanol (10%) at 65°C. The CsA solution was administered at room temperature. The total daily dose of CsA was equally divided over two injections given subcutaneously; the first one was given at about 9.00 a.m. and the second at about 5.00 p.m. In two separate experiments the effects of CsA were investigated on different phases of the disease.

Experiment one. Two rhesus monkeys were used to test the effect of CsA on the arthritic activity when administered during clinically active CIA. CsA was administered when a clear STS of the joint(s) was observed. CsA was injected at a dose of 25 mg/kg per day for 11 or 12 days. This particular dose is well tolerated by rhesus monkeys [9] and proved to be effective in this animal species in inhibiting experimental allergic encephalomyelitis and rejection of a transplanted kidney. Monitoring of CsA-related toxicity and of the anti-CII immune response was not performed in these animals.

Experiment two. Eight rhesus monkeys were used to test the anti-arthritic effect of CsA when administered during the preclinical phase of the disease. The monkeys were divided over two groups of four animals each. In the treatment group the animals received CsA during 25 days: from day 7 to 25 in a dose of 25 mg/kg per day, and subsequently 12·5 mg/kg daily from day 25 to 32. A sham-treatment group of four animals received equivalent volumes (at ml/kg bodyweight) of the Miglyol/ethanol mixture.

At 60 days after immunization CsA-treated animals received a booster immunization with 1·0 mg B-CII in Freund's incomplete adjuvant (FIA) in order to assess if disease exacerbation could be induced [2].

Medication additional to CsA was given at veterinarians' indications to reduce pain or inflammatory activity. Almost all monkeys in the control group of experiment 2 received buprenorphine (Temgesic), a central acting analgesic drug not affecting the reactivity of the immune system [13]. Incidentally, other agents have been given which are also used as antirheumatics in the clinic [14]; from the group of the non-steroidal anti-inflammatory drugs (NSAIDs) phenylbutazone (Prisantol) and acetylsalicylate (Aspesic), and from the group of the corticosteroids cortisone (Diadreson-f-aequosum) and dexamethasone (Dexatomanol). Doses and frequencies of administration are indicated in Table 1.

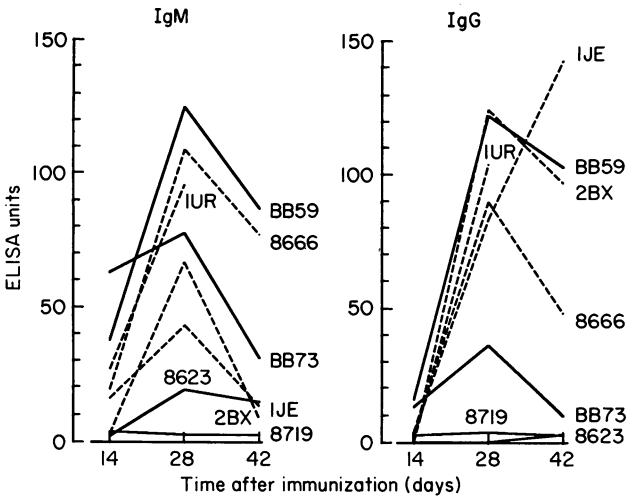

Fig. 1. IgM and IgG antibody levels to type II collagen. At days 14, 28 and 42 after immunization sera were taken and tested for the presence of anti-bovine type II collagen (B-CII) antibodies. Results are given in arbitrary ELISA units, defined as OD at 405 nm. Dotted lines represent data from sham-treated monkeys, whereas data from cyclosporin A (CsA)-treated monkeys are given in solid lines.

Anti-CII antibody responses

Anti-B-CII IgM and IgG antibodies in the monkeys of experiment 2 were measured with ELISA [2]. Blood samples for serum preparations were taken at days −7, 14, 28 and 42. Flexible 96-well assay plates (Falcon 3911; Oxnard, CA) were coated overnight at 4°C with B-CII (10 μg/ml; 50 μl/well). Plates were post-coated with 100 μl 1% bovine serum albumin (BSA) in PBS for 60 min at 37°C. Serum samples (1:200) were added, 50 μl/well, and incubated for 1 h at 37°C. Subsequently, alkaline phosphatase-conjugated goat anti-human IgG or IgM (Tago, Burlingame, CA) was added, 50 μl/well, at a 1:1000 dilution in PBS/1% BSA and the plates were incubated for 1 h at 37°C. As substrate *p*-nitro-phenylphosphate (Sigma Chemical Co., St Louis, MO) was added. Colour development was measured on a Titertek Multiskan Plus Mark II at 405 nm.

Haematology and blood chemistry

Parameters for haematology and blood chemistry were measured every 14 days using standard procedures at the diagnostic laboratory of the Stichting Samenwerkende Delftse Ziekenhuizen (Delft, The Netherlands). The monitored parameters were: (i) haematology: erythrocyte sedimentation rate (ESR), total leucocytes, differential leucocyte counts, erythrocyte count, platelet count, haemoglobin and haematocrit; (ii) clinical chemistry: creatinin, albumin, total protein, urea, alkaline phosphatase, uric acid, glucose and transferrin; and (iii) acute phase reactants: C-reactive protein (CRP), complement factor C3.

RESULTS

Effects of CsA on arthritic activity

In two separate experiments the effect of CsA was investigated on different phases of the disease.

Experiment 1 (Table 1a). Administration of CsA was started at day 7 after immunization, but before onset of the disease. All four sham-treated monkeys (8666, 1JE, 1UR, 2BX) developed the first signs of arthritis between days 21 and 28, followed by a disease period of 8–21 days that was characterized by STS of ankles, wrists and small joints of hands and feet, and by enhanced levels of ESR and CRP. To relieve pain and/or to reduce inflammatory activity, buprenorfine in combination with phenylbutazone, acetylsalicylate or cortisone were given as indicated in the footnotes to Table 1. Based on criteria of the animal's appearance and behaviour, none of these drugs had a beneficial effect on pain, and no reduction of STS in the affected joints could be detected. It was necessary to kill monkey 2BX at the veterinarians' indication.

The four animals (BB59, BB73, 8719 and 8623) in the CsA-treated group lacked any STS of the joints. Three monkeys showed negative ESR values, but slightly elevated levels of CRP. Only monkey BB59 showed a raised ESR level of 48 mm/*l* together with a relatively high CRP level (183 mg/ml). The monitored haematologic parameters (see Materials and Methods) were all in the range found in healthy animals, with the exception of some aberrant values found in monkey BB59.

The CsA-treated monkeys received a booster immunization with B-CII at day 60 after the first immunization. This failed to induce detectable arthritic activity, although at 1 week after the booster immunization slightly elevated ESR values were found in all four animals (data not shown).

Experiment 2 (Table 1b). CsA treatment (25 mg/kg per day) was started after clinical manifestation of disease. In animal 4116 STS was most manifest in the ankles, and in 2774 in the small joints of hands and feet. In both animals the inflammatory activity was reflected by raised levels of ESR and CRP. Administration of CsA was started in 4116 and 2774 respectively 3 and 7 days after onset of disease, but no improvement of the disease was observed. Monkey 4116 received additional medication with buprenorfine and dexamethason, but without any beneficial effect. This animal was killed at day 86 after immunization at the veterinarians' indication. The period of 18 days of arthritic activity in monkey 2774 was similar to the non-treated control animals in Table 1a.

Inhibitory effects of CsA on the antibody response to CII

The inhibitory effect of CsA on the antibody response to B-CII was investigated in the animals in Table 1a. Both IgG and IgM isotype antibodies were measured (Fig. 1). No significantly different anti-CII IgM antibody levels were found between treated and non-treated monkeys. However, except for monkey BB59, the anti-CII IgG levels in the CsA-treated animals at days 28 and 42 were significantly ($P < 0{\cdot}025$) lower than in the sham-treated animals.

Effect of CsA on haematologic parameters

In the animals in Table 1a, CsA administered subcutaneously in a dose regime of 25 mg/kg per day during 9–18 days plus 12·5 mg/kg per day for 7 days proved to have no adverse side effects. In three of the four CsA-treated monkeys, all monitored haematological and clinical chemistry parameters were in the range found in healthy animals. Only in monkey BB59 were some parameters outside the normal range, namely total leucocyte numbers, haemoglobin, haematocrit, albumin and alkaline phosphatase (Table 2a). Comparable values of the same parameters were measured in the sham-treated monkeys during the arthritic period (data not shown). This points at subclinical disease activity in the monkey. This assumption is confirmed by

Table 2. Haematological blood parameters: (a) Blood parameters of rhesus monkey BB59 falling outside the normal range. (b) Serum levels of urea (in mmol/*l*) and creatinine (in μmol/*l*) were monitored as a measure of possible nephrotoxicity in cyclosporin A (CsA)-treated and control animals on days 14, 28, 42 and 63

(a) Parameter*	Day 28	Day 42	Normal range†
Leucocytes ($\times 10^9/l$)	16·3	9·2	3·4–14·3
Haemoglobin (mmol/*l*)	5·2	5·0	6·0–9·1
Haematocrit (*l*/*l*)	0·25	0·24	0·30–0·44
Albumin (g/*l*)	16	31	34–46
Alkaline phosphatase (U/ml)	587	ND	64–414

(b)		Creatinine (μmol/*l*)				Urea (mmol/*l*)			
Monkey	CsA treatment	Day 14	Day 28	Day 42	Day 63	Day 14	Day 28	Day 42	Day 63
8666	No	66	52	56	ND	3·6	6·2	2·1	ND
1JE	No	86	105	86	ND	5·5	6·4	4·9	ND
1UR	No	89	80	ND	ND	5·5	8·0	ND	ND
2BX	No	81	83	44	ND	6·6	9·7	8·5	ND
BB59	Days 7–32	63	87	82	65	6·9	10·8	6·9	5·6
BB73	Days 7–32	93	85	75	66	5·7	6·0	4·6	3·5
8719	Days 7–32	82	109	104	90	5·0	6·0	4·9	6·4
8623	Days 7–32	60	73	67	60	4·2	6·4	5·1	4·8

* Days 28 and 42 correspond with day 21 of CsA administration and day 10 after the CsA administration was terminated.

† As determined for the rhesus monkeys at the TNO-Primate Centre, Rijswijk, The Netherlands.

ND, Not determined.

the lower immunosuppressive effect of CsA found in this animal (Fig. 1). Serum levels of creatinine and urea in the CsA- and sham-treated animals of Table 1a, which are indicative for possible nephrotoxicity of the applied CsA dose, are given separately in Table 2b. These results show no significant differences between the two groups.

DISCUSSION

The results described in this study clearly demonstrate that administration of CsA in the period between induction and onset of CIA suppresses CIA in the rhesus monkey. Based on the monitored blood parameters we conclude that the administered doses of CsA were non-toxic in rhesus monkeys, although subclinical organ pathology cannot be excluded. Of particular relevance is the absence of nephrotoxicity, as could be concluded from the unaltered urea and creatinine levels (Table 2b). Other studies in rhesus monkeys have shown that even at an oral dose of 200 mg/kg per day given for 13 weeks, toxicity of CsA is minimal [15]. The anti-arthritic activity of CsA is reflected by a reduction of anti-CII IgG antibody production. In contrast to studies in rodent species, in which arthritis could be induced in naive recipients by transfer of T cells from immunized animals [16], an effector function of T cells in rhesus monkey CIA has been demonstrated only in an indirect manner. In a comparative study comprising CIA-resistant and CIA-susceptible monkeys, evidence was provided that anti-CII autoantibodies, particularly those of the IgM isotype, together with CII-reactive T cells, are essential in the induction phase of the disease, while the possibility of inducing a flare-up is regulated at the T cell level [2,3,17,18]. The inhibition of CIA by CsA confirms that T cells play an important pathogenic role in rhesus monkey CIA. However, although clinically manifest CIA is reflected by high T cell proliferative responses to CII *in vitro* [3], CsA did not suppress clinically manifest CIA, but could only prevent the onset of CIA. The observation that prophylactic treatment with CsA effectively prevents the development of CIA, whereas therapeutic treatment is ineffective, is in agreement with data obtained in rodents [19,20]. Such observations indicate that either the pathogenic T cells are insensitive to CsA, or that T cells are involved in the induction of CIA, but might be less important during the active period of the disease.

Of particular interest for understanding the pathogenesis of CIA in rhesus monkeys is the observation that CsA-treated animals become resistant to a renewed disease induction. Taking into account (i) that IL-2 is an indispensable costimulatory factor in CII-reactive T cell activation [18], and (ii) that CsA suppresses IL-2 release [21], this observation indicates that specific induction of tolerance or anergy [22] might have taken place.

In clinical practice it is generally experienced that similar to the situation in experimental models, the activity of CsA is highest when the drug is administered during the induction phase of an immune response. Nevertheless, clear differences exist between the response of rhesus monkey CIA and human arthritis diseases to CsA. Therapeutic effects of relatively low doses of CsA were reported on clinically active relapsing polychondritis (3 mg CsA/kg per day) [12] and rheumatoid arthritis (5 mg CsA/kg per day) [11], although the duration of the therapy was longer in the patient studies. Interestingly, in

clinically active rheumatoid arthritis, CsA treatment has no effect on ESR values, but acute phase proteins, such as CRP, are decreased during CsA therapy. In contrast, prophylactic treatment with CsA of rhesus monkey CIA decreases ESR as well as CRP values.

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