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Preventive and therapeutic effects of cyclosporin and valine^2-dihydro-cyclosporin in chronic relapsing experimental allergic encephalomyelitis in the Lewis rat

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SUMMARY

Cyclosporin (CS) and valine^2-dihydro-cyclosporin [(Val^2)DH-CS] were tested in adult Lewis rats with chronic relapsing experimental allergic encephalomyelitis (CR-EAE), induced by the immunization of guinea-pig spinal cord emulsified in complete Freund's adjuvant. The drugs were given orally for 15 or 35 days, at 12·5, 25 or 50 mg/kg/day, starting either on the day of sensitization (preventive treatment) or at one of three subsequent times (therapeutic treatment): the onset of the first attack (protocol A); the onset of the first spontaneous remission (protocol B); and the onset of the second attack (protocol C). Used therapeutically in protocol A, at doses above 12·5 mg/kg/day, both drugs prolonged remission past the end of therapy in more than two-thirds of the treated animals, compared to $< 10\%$ of controls. Trends were similar under protocols B and C. Disease developing after preventive treatment with either drug was predominated by chronic and hyperacute attacks, in contrast to the relapsing course of controls. This pattern was also the result after CS was given therapeutically, whereas (Val^2)DH-CS in such circumstances eliminated all further attacks in the majority of rats (58–86% at 25 mg/kg/day) and only minimal disease occurred in the remainder. We conclude that both drugs, in this model, are beneficial during administration; however, in contrast to CS, (Val^2)DH-CS possesses an important, curative action when applied therapeutically.

INTRODUCTION

Experimental allergic encephalomyelitis (EAE) has been produced in many animal species and serves as the classical model of autoimmune disease in the central nervous system (Paterson, 1966). Its chronic relapsing form, in particular, offers important similarities to the human disease multiple sclerosis (MS) (Wisniewski & Keith, 1977; Lassman, 1983a; McFarlin & McFarland, 1982). A recently developed model of chronic relapsing EAE (CR-EAE) in the Lewis rat has highly reproducible features, such as the number, timing and severity of attacks (Feurer, Prentice & Cammisuli, 1985). This should be well suited to the laboratory study of MS, including its therapy in various stages of the disease.

Cyclosporin (CS) is a selective and powerful immunosuppressive agent, which is widely used to prevent organ rejection after transplantation (Kahan, 1983; Beveridge, 1986). Its use in chronic inflammation and autoimmunity is currently under extensive study in both animals and patients (Borel & Gunn, 1986; Schindler, 1985; Hinrichs, Wegmann & Peters, 1983; Bolton *et al.*, 1982b; Bolton, Allsopp & Cuzner, 1982a; Reiber & Suckling, 1986). Valine^2-dihydro-cyclosporin [(Val^2)DH-CS] is a newer analogue, with a more restricted immunosuppressive profile. It produces benefit primarily in states of cell-mediated hypersensitivity, including certain autoimmune conditions (Borel *et al.*, 1986; Hiestand *et al.*, 1985; Cammisuli & Feurer, 1984).

In the present study, we tested the prophylactic and therapeutic effects of CS and (Val^2)DH-CS in CR-EAE in the Lewis rat. We found important differences between these drugs, especially in the way they affected the course of disease after stopping drug administration.

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MATERIALS AND METHODS

Animals

Female Lewis rats, approximately 8 weeks old, were obtained from Mollegaards Breeding Centre, Ejby, Denmark. They were kept in conventional quarters and maintained under standard conditions. Diseased rats were provided with easy access to food and water and maintained in a dry condition.

Induction of EAE

The procedure has been described previously (Feurer *et al.*, 1985). Briefly, guinea-pig spinal cord (GPSC) was emulsified in Difco's Bacto complete adjuvant H37RA, supplemented with additional *Mycobacterium tuberculosis* H37RA (Tbc) (Difco

Laboratories, Detroit, MI). Rats were inoculated intradermally with a total of 0·2 ml of the emulsion, divided equally between both hind footpads, giving 58 mg GPSC and 2·2 mg Tbc per animal.

Clinical assessment of EAE

Rats were observed daily for signs of EAE and were scored as follows: 0, normal; 1, hypotonic tail; 2, weakness of at least one hindlimb, or mild ataxia; 3, paralysis of distal hindlimbs, or severe ataxia; 4, complete hindlimb paralysis accompanied by urinary incontinence. For purposes of analysis, disease with a minimum intensity of grade 2 was considered as an attack.

Each experiment lasted 70–100 days. The course of EAE in each animal was classified as follows: monophasic, a single attack, leading to complete recovery in 7–10 days without further disease; relapsing, more than one bout of disease, separated by remissions at grade 0 or 1; chronic, sustained disease, with occasional short-lasting improvements barely affecting the grade; hyperacute, rapidly progressive disease up to grade 4, with death within 2–10 days.

To allow quantification of a single attack in an animal, a severity index (SI) was derived as follows:

$$SI = (\text{average grade of EAE} - 1) \times (\text{duration of attack}).$$

This was analogous to calculating the area under the curve of a mathematical function. In the above expression, the first factor of the index was weighted so as to discount disease of grade 1 intensity. In addition, chronic attacks may be over-represented solely due to their length of follow-up; accordingly, application of the index was restricted to the first 10 days of such cases. Conversely, an arbitrary score of 15 was assigned to a hyperacute attack, to eliminate the under-representation of early mortality.

Treatment protocol

CS or (Val²)DH-CS was dissolved in pure ethanol and then mixed with olive oil, yielding a final ethanol concentration of 10%. Drug doses were 12·5, 25 or 50 mg/kg/day. These dosages have been found effective in other rat models of autoimmunity or chronic inflammation (Borel *et al.*, 1986). Some rats received solvent alone, at 5 ml/kg/day, equivalent to the volume used to deliver the drugs. All treatments were administered by gastric tube. When used prophylactically, therapy was begun on the day of sensitization, and continued for a total of 15–35 days, as specified in the text. On the other hand, treatment given therapeutically was administered for 15 days, commencing at one of three time points: the onset of the first attack, the onset of the first spontaneous remission, and the onset of the second attack. For this purpose, the first attack was deemed to begin when its intensity reached or exceeded grade 3, the typical starting score previously (Feurer *et al.*, 1985) found in untreated, control rats.

Statistical analysis

Comparison of means was performed using the Student's *t*-test, while the comparison of frequencies employed chi-square.

RESULTS

Controls

The first sign of EAE in untreated control rats appeared at 11·0±0·8 (mean±SD) days post-immunization (PI), affecting all 47 of the immunized animals. One rat in this group developed chronic EAE, but the remainder recovered, achieving remission at 17·6±1·0 days PI. The SI for this attack was 11·3±2·5, with two-thirds of the group reaching a peak intensity of grade 4. A second attack was found in 96% of those who remitted, beginning at 19·2±1·7 days PI. Compared to the first, this episode was generally less severe, having an SI of 6·5±4·1 ($n=44$), and full remission followed in all animals by 24·9±2·9 days PI. Forty-five per cent of them later manifested a third attack, at 32·8±9·5 days PI, which again remitted after several days, giving an SI of 2·9±1·8 ($n=20$).

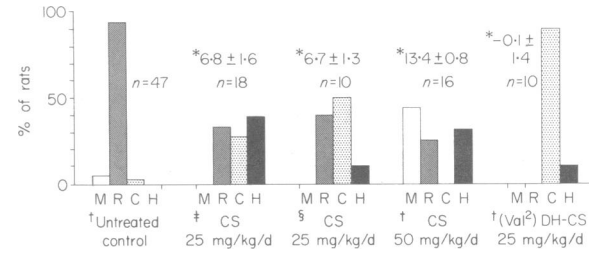

Figure 1. Pattern of disease following preventive treatment. EAE developed in all rats following cessation of drug therapy. The pattern of disease is shown. There was a shift from the relapsing disease of control animals to chronic and hyperacute forms in animals after drug treatment. M, monophasic; R, relapsing; C, chronic; H, hyperacute (d, day). * Mean disease-free interval (days±SD) after cessation of drug therapy. † Control rats developed their first attack at 11·0±0·8 days post-immunization. ‡ Drug given on Days 0–14 inclusive. § Drug given on Days 0–34 inclusive.

An additional 18 control rats were immunized, and these were treated with solvent at the onset of the first attack. This occurred at 11·1±0·7 days PI, with 100% incidence. Daily treatment was instituted for 15 days. The course of EAE in these vehicle-treated animals was similar to that of untreated controls: the SI for the first attack was 10·3±2·2; all 18 animals achieved remission after approximately 5 days, and then each one relapsed within the period of olive oil administration. The SI for the second attack was 6·1±3·0. Six of the 18 rats subsequently experienced a third attack with an SI of 3·7±1·6.

Preventive treatment

Starting on the day of immunization (Day 0), a 15-day course of the following was given: CS or (Val²)DH-CS at 25 mg/kg/day ($n=18$, $n=10$, respectively); and CS at 50 mg/kg/day ($n=16$). An initial attempt to use (Val²)DH-CS at 50 mg/kg/day was abandoned because of reversible toxicity which became apparent after the first week of treatment. In another group of rats, therapy with CS at 25 mg/kg/day was extended from Day 0 to Day 34 inclusive ($n=10$).

The data in Fig. 1 show that all rats developed EAE following cessation of treatment. When CS was given at 25 mg/kg/day, whether it was for 15 or 35 days, there was no sign of EAE until an average of 1 week after stopping therapy. At 50 mg/kg/day, this period of protection was further extended to a total of about 13 days. (Val²)DH-CS delayed the onset of EAE by only 3 days, relative to controls, with protection elapsing by the end of treatment.

Figure 1 also displays the pattern of disease that followed therapy. Both drugs at 25 mg/kg/day resulted in a high

Table 1. Complete remission on therapeutic treatment of CR-EAE

Drug	Dose (mg/kg/day)*	(*n*)	% with complete remission	No. of days from start of treatment	% with substained remission	
					To end of treatment	To end of study†
Protocol A: *Treatment beginning at onset of first attack*						
Untreated control		47	98	6·2±0·8‡	9§	4
Vehicle-treated control		18	100	5·2±0·9	0	0
CS	12·5	10	90	6·6±0·5	10	10
	25	18	100	5·4±0·8	67¶	6
	50	18	100	4·3±0·8	94¶	0
(Val²)DH-CS	12·5	11	100	5·5±0·7	9	9
	25	19	100	4·9±0·7	74¶	58¶
Protocol B: *Treatment beginning at onset of first spontaneous remission*						
Untreated control		46			9	4
CS	25	7			71¶	0
(Val²)DH-CS	25	8			88¶	75¶
Protocol C: *Treatment beginning at onset of second attack*						
Untreated control		44	100	5·3±2·6‡	64§	55
CS	25	7	100	3·4±1·7	71	0**
(Val²)DH-CS	25	7	100	3·1±2·0	100	86

* Treatment given daily for 15 consecutive days by gastric tube.
† Observation period ranged from 2 to 3 months.
‡ Entry indicates no. of days from onset of attack (mean±SD).
§ Percentage of controls with remission sustained ⩾15 days from onset of attack.
¶ Significantly different than control, $P<0·01$.
** Significantly different than control, $P<0·05$.

percentage of chronic or hyperacute attacks, in contrast to the normal, relapsing course of control animals. At 50 mg/kg/day, CS produced a monophasic disease in nearly 50% of cases, in addition to the hyperacute form in the same group.

Therapeutic treatment

Treatment given for 15 days at the onset of the first attack (protocol A) consisted of the following: CS at 12·5, 25 and 50 mg/kg/day ($n=10$, $n=18$, $n=18$, respectively); and (Val²)DH-CS at 12·5 and 25 mg/kg/day ($n=11$, $n=19$, respectively). Subsequently, a similar course of CS or (Val²)DH-CS was tested, at 25 mg/kg/day, beginning at the onset of the first spontaneous remission (protocol B) ($n=7$ for CS; $n=8$ for (Val²)DH-CS), or at the start of the second attack (protocol C) ($n=7$ for each drug).

The results are summarized in Table 1. Under protocols A and C, complete remission during treatment occurred almost invariably after 3–7 days, as it also did, spontaneously, in control animals after 5–6 days. However, more than 90% of the controls in protocol A relapsed within an average of 1·6±0·9 days after the onset of first remission, whereas, as Table 1 shows, a dose-dependent percentage of drug-treated rats sustained their first remission beyond the end of therapy: at 12·5 mg/kg/day, both CS and (Val²)DH-CS were subtherapeutic; at 25 mg/kg/day, two-thirds to three-quarters of the animals were free from relapse during treatment, and this figure rose to 94% when CS was given at 50 mg/kg/day. Furthermore, relapses that occurred during treatment were conspicuously mild (data not shown). For example, for CS and (Val²)DH-CS at 25 mg/kg/day, the average SI was 2·0±0·9 and 1.8±0·8, respectively, which was substantially lower than the value of 6·5±4·1 calculated from controls during a natural, second attack ($P<0·05$).

Benefit was likewise evident in protocol B, where 71% (CS) and 88% [(Val²)DH-CS] of the rats did not experience a second attack during treatment, representing an eight- to 10-fold advantage over controls (Table 1).

Improvement under protocol C, in this regard, was less convincing, especially for CS, owing to the more prolonged remission in control rats before their third attack. Nevertheless, the complete absence of relapse during treatment with (Val²)DH-CS was noteworthy.

Differences between CS and (Val²)DH-CS emerged more dramatically after termination of treatment. The percentage of rats which were able to sustain their remission indefinitely is indicated in Table 1. Almost all animals relapsed after CS administration, regardless of the dose or the time of treatment. In marked contrast, at its therapeutic dose of 25 mg/kg/day, (Val²)DH-CS abolished all further disease in over half of the rats in protocol A, and in three-quarters or more of them in protocols B and C.

As shown in Table 2, there was also a marked difference in the severity of relapses occurring after therapy was stopped. With increasing dosage of CS, as found in protocol A, such relapses actually worsened. When both drugs were given at 25 mg/kg/day under protocol A, the SI of relapses after (Val²)DH-CS was about one-tenth of the value obtained with CS ($P<0·01$). This striking difference persisted in protocols B and C.

Moreover, these drugs had opposite influences on the subsequent pattern of EAE, as illustrated in Fig. 2 for protocol A. As with preventive therapy, CS given therapeutically under

Table 2. Severity of relapse after end of treatment

Drug	Dose (mg/kg/day)*	(*n*)†	Severity index‡ (mean ± SD)
Protocol A: *Treatment beginning at onset of first attack*			
CS	12·5	8 (10)	5·1 ± 4·6
	25	17 (18)	11·1 ± 5·6§
	50	18 (18)	14·1 ± 4·7§
(Val²)DH-CS	12·5	1 (11)	1
	25	5 (19)	1·2 ± 0·4¶
Protocol B: *Treatment beginning at onset of first spontaneous remission*			
CS	25	7 (7)	9·0 ± 5·2
(Val²)DH-CS	25	1 (8)	1
Protocol C: *Treatment beginning at onset of second attack*			
CS	25	7 (7)	11·7 ± 4·2**
(Val²)DH-CS	25	1 (7)	2

* Treatment was given daily for 15 consecutive days, by gastric tube.

† Only animals showing a relapse after completion of treatment are included; the size of the original group is given in parentheses.

‡ See the Materials and Methods for details of calculation.

§ More severe than the second attack in untreated controls (SI = 6·5 ± 4·1, $n = 44$), $P < 0.01$.

¶ Less severe than the second attack in untreated controls, $P < 0.01$.

** More severe than the third attack in untreated controls (SI = 2·9 ± 1·8, $n = 20$), $P < 0.01$.

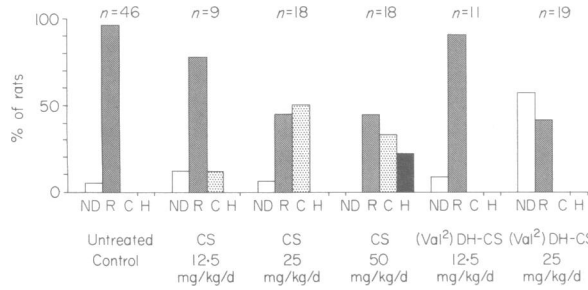

Figure 2. Pattern of relapse following treatment of first attack. Treatment was given for 15 consecutive days beginning at the onset of the first attack. Animals which did not first attain a remission are not considered, representing 0–10% in the various groups. There was a tendency towards chronic and hyperacute forms after CS therapy, as opposed to relapsing or no disease after (Val²)DH-CS. ND, no disease; R, relapsing; C, chronic; H, hyperacute (d, day).

protocol A produced a predominance of chronic and hyperacute forms. This trend, which was already suggested at the subtherapeutic dose of 12·5 mg/kg/day, became quite clear at higher dosages. No such deleterious effect was detected after (Val²)DH-CS therapy, which, in contrast, eliminated all further disease in the majority of animals given 25 mg/kg/day, as already pointed out. The same distinctions were observed under protocols B and C (Fig. 3). However, a large proportion of CS-treated animals followed a natural, relapsing course when the drug was introduced during spontaneous remission (protocol B), as opposed to CS administration begun at the onset of the first or the second attack (protocols A and C).

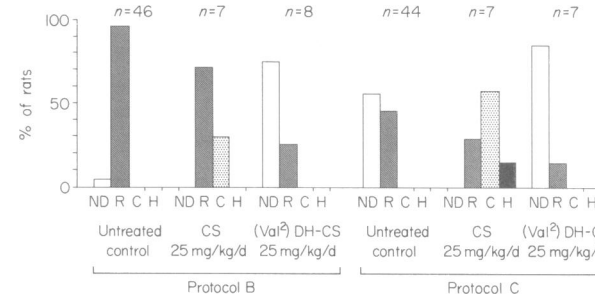

Figure 3. Pattern of relapse following treatment at first remission or at second attack. Treatment was given for 15 consecutive days, beginning at the onset of the first spontaneous remission (protocol B), or at the onset of the second attack (protocol C). Under both protocols, there was a shift towards chronic and hyperacute forms after CS therapy, but towards no disease after (Val²)DH-CS. ND, no disease; R, relapsing; C, chronic; H, hyperacute (d, day).

DISCUSSION

CR-EAE in the Lewis rat was suppressed by CS or (Val²)DH-CS, administered either preventively or therapeutically, in agreement with data obtained previously with several models of chronic inflammation (Borel *et al.*, 1986). Treated preventively in CR-EAE, disease ultimately emerged in every animal, with a length of quiescence that depended on the drug and the dose employed. Thus, for CS at 25 mg/kg/day, given for up to 35 days, disease followed cessation of therapy by an average of 1 week. This duration post-treatment does not seem sufficient for EAE to develop *de novo*, a process which required 11·0 ± 0·8 days in control rats. It follows that the effector mechanism, although clearly blocked at some level, was already activated during therapy. Similar activation during therapy has been demonstrated in other models (Chisholm *et al.*, 1985). According to the scheme of Hinrichs, Roberts & Waxman (1981), such activation means that precursor effector cells were sensitized, which might also provide an explanation for the exaggerated disease which followed post-treatment. In other words, effector precursors, activated but prevented from maturation, might accumulate to very large numbers, to be released all at once upon drug withdrawal, causing a predominance of hyperacute and chronic forms of EAE.

For CS at 50 mg/kg/day, clinical disease was delayed until 13 days post-treatment, long enough to infer *de novo* antigen recognition. This may correspond to the high-dose suppression demonstrated for CS in human mixed-lymphocyte reactions, where the effector mechanism was blocked directly at the level of precursor sensitization (Hess, 1985). In this case, with high-dose therapy, there must be a different explanation for the enhanced EAE post-treatment. Such an explanation might be that substantial, uncontrolled changes in composition occurred within the intradermal antigen depot during the 15-day delay in sensitization imposed by therapy. These changes would drastically alter the subsequent expression of EAE (Lassmann, 1983b; Feurer *et al.*, 1985).

A further explanation for enhanced disease post-treatment, applicable to both high and low dosages of CS used, is that critical suppressor mechanisms might have been damaged

(Hinrichs *et al.*, 1981; Welch, Holda & Swanborg, 1980). By speculation, the suppressor arm of the immune reponse may have a particularly strategic role in relapsing disorders, such as CR-EAE and MS, where a changing equilibrium between these and effector cells might account for the repetitive disease fluctuations observed.

For animals receiving a 15-day course of (Val²)DH-CS preventively, clinical EAE began, on the average, on the last day of treatment, and assumed a notably chronic course. It is important to bear in mind that the earlier onset of disease, compared to CS, might actually reflect a different mode of action for (Val²)DH-CS. Both agents are expected to act by interfering with lymphokines. However, (Val²)DH-CS does not prevent antibody formation, functions poorly in models of organ transplantation, and exerts its strongest effects in delayed-type hypersensitivity and other settings of chronic inflammation (Borel *et al.*, 1986). Differences in the basic action of the two drugs become especially relevant in the therapeutic phase of our study, in which the late effects of CS and (Val²)DH-CS sharply contrasted.

Under these therapeutic protocols, both compounds were efficacious during treatment. However, whereas CS therapy again led to exacerbated disease post-treatment, (Val²)DH-CS exerted a lasting, curative action, as evident in most animals. After (Val²)DH-CS treatment at 25 mg/kg/day, 58–86% of rats were completely free of relapse for the entire observation period, extending up to 10 weeks post-treatment, while the remainder experienced only minimal disease, as reflected in their SI scores.

This effect of (Val²)DH-CS is divergent from results of the drug's preventive usage (Fig. 1), and no clear explanation is possible from existent data. However, although apparently paradoxical, the situation is not irrational. Significant differences in immunological milieu exist between preventive and therapeutic phases of our study. Specific suppressor cells, which should be essentially absent during antigen presentation, are well developed by the beginning of the first attack (C. Feurer, unpublished observations), when the earliest therapeutic regiment commences. By purposely allowing the initial progression of disease, therapeutic protocols also deal with a wholly different mixture of precursor and mature effector cells. Furthermore, effector memory cells, hypothesized by some to be principally responsible for reactivation of disease (Willenborg, Sjollema & Danta, 1986), might be non-existent when preventive therapy has blocked the differentiation of effector precursors from the start, but present and susceptible to lasting inhibition when therapy is delayed.

The results of therapeutic protocols A and C suggest another point about CS and (Val²)DH-CS therapy, namely, that both are ineffective against fully differentiated effector cells in this model. Different dosages of the drugs failed to significantly shorten the time required to arrest an on-going attack. At the highest dosages, 3 to 4 days delay remained. This is consistent with the need for terminal effectors to exhaust themselves spontaneously. Such cells demonstrate self-limited activity lasting 4–5 days when studied in the model of acute, monophasic EAE (Willenborg *et al.*, 1986).

We have begun to examine the kinetics of suppressor and effector cells in CR-EAE in the Lewis rat. Better knowledge in this direction may help resolve yet unanswered questions concerning CS and (Val²)DH-CS therapy in this model of relapsing disease.

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