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Cyclosporine A versus cyclosporine G: a comparative study of survival, hepatotoxicity, nephrotoxicity, and splenic atrophy in BALB/c mice

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Abstract. Our group has previously shown that cyclosporine A (CSA) but not cyclosporine G (CSG) causes splenic atrophy in a BALB/c mouse model. We have now extended our studies to observations of the effect of the two drugs on other parenchymal organs and on the nervous system. Groups of mice ($N=30$) were given 150 mg/kg per day of either CSA or CSG and were compared to two control groups. Absorption of the drugs was similar in the two groups, although CSG blood levels were slightly higher. Animals treated with CSA, but not CSG, lost up to 50% of body weight over a 3-week period. Overall mortality was much higher in the CSA group. Blood urea levels were significantly higher in both treatment groups than in controls and were significantly higher in the CSA than in the CSG group. CSA-treated animals showed marked histological changes in their kidneys, the most prominent of which was proximal tubular vacuolation. Both drugs showed some hepatotoxicity, both histologically and biochemically; the histological changes were more marked in the CSA group. There was no pancreatic toxicity at this dose, either histologically or in terms of blood-sugar concentrations. Mice treated with CSA, but not with CSG, showed marked behavioral changes, including hyperactivity and irritability. The most intriguing observation was the effect of CSA, but not CSG, on the spleen. There was atrophy of lymphoid tissue in both the B and the T cell areas, although the most prominent change was in the periarterial lymphatic sheaths. These changes may be of significance in the long-term maintenance of immunosuppression and graft acceptance. CSG appears, therefore, to be significantly less toxic overall in this model than CSA and warrants further study, both experimentally and clinically.

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Cyclosporine A (CSA) has enjoyed spectacular success as an immunosuppressive agent in clinical transplantation. Eleven years after the demonstration of its immunosuppressive properties [4], and 8 years after its first tentative clinical use in Cambridge, England [5], it is widely used around the world, although we are still learning how best to use the drug. CSA was not considered to be a totally safe drug at the beginning, nor is it considered safe now. The list of potential clinical complications is long [16], and clinically the most important of these is its nephrotoxicity, at least in the short term. However, CSA also has a deleterious effect on other organs. In a recent review by Kahan et al. [16], for example, up to 50% of the patients studied had an element of hepatic dysfunction. The drug is also known to affect the pancreas, both in experimental animals and in man [18, 22].

Cyclosporine G (CSG) (Nva^2-Cyclosporine) was first introduced in 1984 [13] as an analogue equipotent to CSA in immunosuppressive capacity but potentially less nephrotoxic, as indicated by early animal experiments [7, 12]. CSG differs from CSA in that it has norvaline instead of aminobutyric acid in position 2 of the cyclic peptide [27].

In our previous studies with CSG, we have shown that in the BALB/c mouse model [19], CSG is less nephrotoxic than CSA in doses of 50 and 150 mg/kg per day. In the course of those experiments, we noticed that at the higher dose CSA - but not CSG - caused splenic atrophy [8].

Because of the limited number of experimental animal studies with CSG, we decided to extend our studies comparing CSA with CSG in this mouse model. In these experiments, we have used a dose that is deliberately in excess of the immunothera-

peutic range yet within the range in which rodent toxicity studies have been carried out by various groups [7, 23, 27, 28]. The oral LD_{50} dose for CSA in mice is 2300 mg/kg. Our aim was to study the effect of the two drugs on various organs and, moreover, to study the potentially biologically important phenomenon of splenic atrophy [8].

Materials and methods

Experimental groups

A total of 120 BALB/c mice (over 4 months old, bred in our laboratory, original stock obtained from Bantin and Kingman, Alborough, UK) were divided into four groups, each group comprising 30 animals. Those in group I were given CSA at 150 mg/kg per day. Those in group II received 150 mg/kg per day of CSG. Group III received an equivalent volume of the vehicle in which the drugs were dissolved (i.e., a 9:1 olive oil:ethanol solution).

Group IV served as a control group; like the other animals, they were allowed water and food ad libitum but received no treatment. Both CSA and CSG were supplied in powder form (Sandoz, Basle, Switzerland). All mice were kept in a temperature-controlled room. All injections were given subcutaneously. The animals were weighed before the start of treatment and at the end of each week after treatment and observed daily for any physical or behavioral changes.

At the end of each 7-day period, blood was obtained for urea, cyclosporine blood levels, and liver function tests, usually from 6 animals in each group. At this stage, six animals were killed and the spleen, liver, kidneys, and pancreas removed.

Urea measurements

Urea levels were determined on an Astra 8 machine (Beckman Instruments).

Liver function tests

Liver function tests (total protein, albumin, and bilirubin) were carried out on a DACOS (Coulter Diagnostics).

Histology

Thin sections were cut from paraffin-embedded tissue and routinely stained for hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and with silver stain for reticulin.

Cyclosporine levels

Blood levels of CSA and CSG were determined by radioimmunoassay, using kits supplied by Sandoz (Basle, Switzerland).

Table 1. Mean urea levels in mg/dl ± standard deviation. Mean urea levels in mg/dl of mice treated with 150 mg/kg per day of either CSA or CSG as compared to those mice receiving vehicle only or no treatment

Treatment/ week	CSA (I)	CSG (II)	Vehicle (III)	Nil (IV)
1	169.2 ± 106.42[a]	60.4 ± 14.97[b]	32.5 ± 5.97	32 ± 7.54
2	227.3 ± 174.42[a]	127 ± 59.80[b]	31.8 ± 6.42	36 ± 5.65
3	204[a]	70 ± 18.19[b]	43.3 ± 10.07	39.3 ± 10.70

[a] Group statistically different from groups II, III and IV ($P = 0.025$, pooled t-test)
[b] Group statistically different from groups III and IV ($P = 0.025$, pooled t-test)

Table 2. Mean spleen weight in mg ± standard deviation. Mean spleen weight in mg of mice treated with 150 mg/kg per day of either CSA or CSG as compared to those mice receiving vehicle only or no treatment

Treatment/ week	CSA (I)	CSG (II)	Vehicle (III)	Nil (IV)
1	108 ± 15.64[a]	128.11 ± 9.14	123.22 ± 4.25	122.5 ± 5.2
2	59.77 ± 39.36[a]	124.44 ± 9.82	122.44 ± 10.87	122.6 ± 7.56
3	30	149.77 ± 22.8[b]	127.11 ± 9.80	126.66 ± 9.64

[a] Group statistically different from groups II, III and IV ($P = 0.025$, pooled t-test)
[b] Group statistically different from groups III and IV ($P = 0.025$, pooled t-test)

Table 3. Liver function tests

Treatment/ week	CSA (I)	CSG (II)	Vehicle (III)	Nil (IV)
A Mean albumin g/dl ± standard deviation[a]				
1	1.75 ± 0.4[b]	1.86 ± 0.5[b]	2.63 ± 0.5	2.52 ± 0.3
2	1.82 ± 0.6[b]	1.67 ± 0.5[b]	2.63 ± 0.04	2.53 ± 0.4
3	1.73 ± 0.5[b]	1.89 ± 0.3[b]	2.51 ± 0.05	2.51 ± 0.3
B Mean total bilirubin mg/dl ± standard deviation[c]				
1	1.2 ± 0.2	1	1	1
2	1.4 ± 0.8	1.3 ± 0.5	1	1
3	1.8 ± 0.7	1.5 ± 0.4	1	1

[a] Mean albumin blood levels of mice treated with 150 mg/kg per day of either CSA or CSG as compared to control groups
[b] Group statistically different from groups III and IV ($P = 0.025$, pooled t-test)
[c] Mean total bilirubin mg/dl of mice treated with 150 mg/kg per day of either CSA or CSG as compared to control groups

Results

Survival

A total of 17 animals receiving CSA (group I) died. Of these, 7 died in the 1st week, 8 in the 2nd week, and 2 in the 3rd week. In the group receiving CSG (group II), a total of 5 animals died. Of these, 2 died in the 1st week, 2 in the 2nd week, and 1 in the 3rd week. No animals died in groups III and IV. Cyclosporine blood levels of mice receiving CSA were slightly lower than the blood levels of mice receiving CSG. The levels ranged from 1975 to 2700 ng/ml.

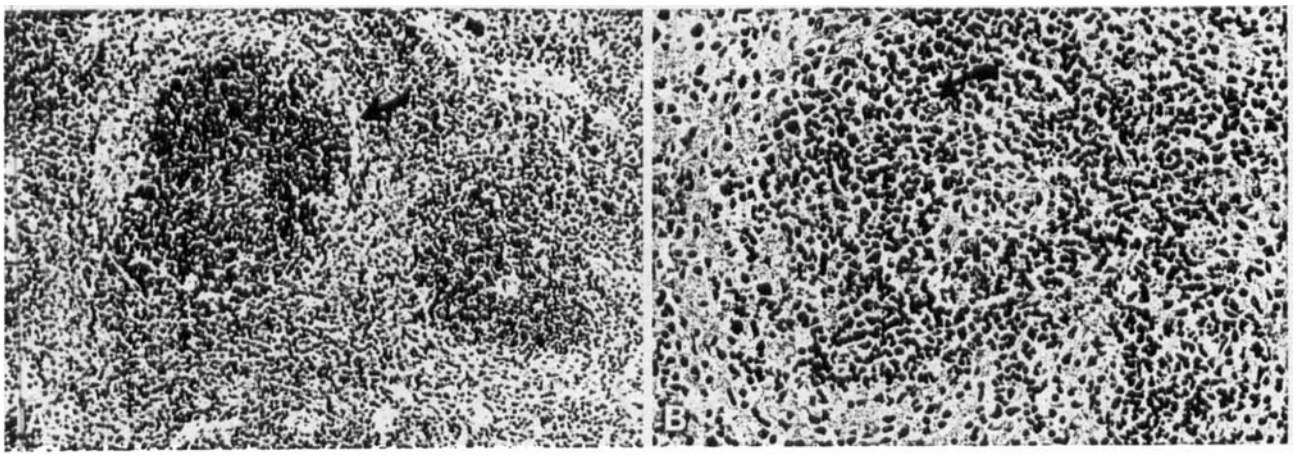

Fig. 1. A Spleen tissue section of a mouse treated with 150 mg/kg per day CSA for 14 days showing the reduced PALS, shrunken white pulp, and the paler areas around the PALS *(arrow)*. B Spleen tissue section of the same mouse showing pyknotic changes and nuclear debris *(arrow)*

Urea levels

CSA caused a sharp rise in the urea levels by the end of the 1st week. This trend continued through the end of the 2nd week. In the 3rd week, a slight drop was observed. CSG, on the other hand, caused a milder elevation in the urea levels, which reached a peak in the 2nd week and then, by the end of the 3rd week, dropped back to the levels observed in the 1st week (Table 1). The rise in urea levels of both CSA- and CSG-treated animals was statistically significantly different from levels in the control groups. The mean urea levels in the CSA group were also significantly higher than in the CSG group.

Spleen weight

Animals treated with CSA showed a significant reduction in spleen weight by the end of the 1st week, a trend which continued into the 2nd and 3rd weeks. Mice treated with CSG for 2 weeks had no change in spleen weight; however, by the end of the 3rd week, there was a significant increase in their spleen weight. Mice treated with the vehicle only had no significant changes in spleen weight (Table 2).

Liver function tests

In both CSA- and CSG-treated animals, there was a significant reduction in albumin levels after 2 weeks. There was also an increase in total bilirubin in the CSA- and CSG-treated animals, but this was not statistically significant (Tables 3 A, B).

Blood glucose levels

Blood glucose levels of animals receiving CSA and CSG were comparable to those of control animals.

Body weight

CSA-treated mice lost 20%–30% of their body weight by the end of the 1st week. This loss increased to 50% by the end of the 2nd and 3rd weeks. CSG-treated mice lost about 10% of their body weight by the end of the 3-week period, while control animals gained about 10%–15% by the end of the same period.

Histology

Liver. At the end of the 1st week, there was no demonstrable change in the livers of mice treated with either CSA or CSG. These livers were comparable to those of mice receiving the vehicle only or those having received no treatment.

By the end of the 2nd week, mice treated with CSG exhibited mild centrilobular congestion and mild fatty changes around central veins. In animals receiving CSA, additional changes in morphology could be observed, among them: (1) intralobular congestion, (2) cells around the central veins which appeared to be smaller than normal, and (3) the presence of lymphocytes in the sinusoid.

Spleen. The spleens of CSA-treated mice showed significant changes. First, there was a marked reduction in the periarterial lymphatic sheath (PALS) area (Fig. 1 A), and pyknotic changes and nuclear debris in PALS could be seen. Then, the entire white pulp of the spleen had atrophied, resulting in prominence of the red pulp, which contained cellular debris. The red pulp was, nevertheless, less cellular than normal (Fig. 1 B). Furthermore, there was a reduction in cellularity around the PALS zones.

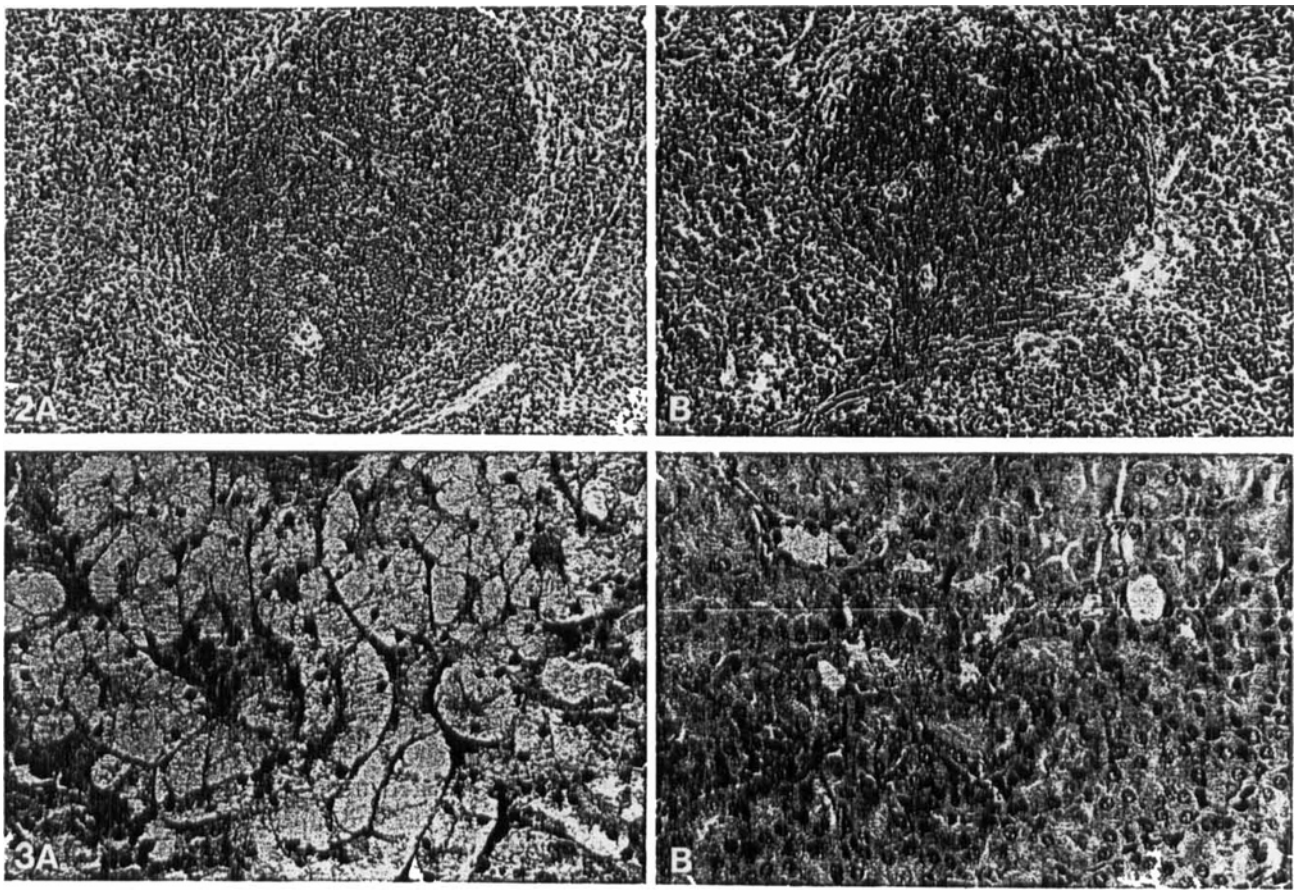

Fig. 2. A Spleen tissue section of a mouse treated with 150 mg/kg per day CSG for 14 days showing normal red pulp and normal PALS. B Spleen tissue section of a mouse treated with 150 mg/kg per day CSG for 21 days showing an increase in cellularity of red pulp area and increase in PALS

Fig. 3. A Kidney tissue section of a mouse treated with 150 mg/kg per day CSA for 14 days showing a marked vacuolation in the proximal tubules. B Kidney tissue section of a mouse treated with 150 mg/kg per day CSG for 21 days showing normal proximal tubules

This may suggest a possible arrest in the normal migration of lymphocytes. Finally, wear and tear pigments and hemosiderin in both the white and red pulp were observed. Mice treated with CSG for 2 weeks had normal spleen histology. However, by the end of the 3rd week, there was an expansion of the red pulp area and an increase in the size of PALS (Fig. 2A, B).

Kidney. After 1 week of treatment, CSA-induced tubular lesions that are characteristic of CSA nephrotoxicity in man and in rats were observed. These changes include dilatation of proximal tubules, moderate peritubular congestion, and marked vacuolation of the cytoplasm in the cells of the straight segment of the proximal tubules and the juxtaglomerular apparatus (Fig. 3A, B).

Mice treated with CSG, on the other hand, had only mild dilation of the tubules after 1 week of treatment. Mild peritubular congestion was seen at the end of 2 weeks. Mild vacuolation was detected after 3 weeks of treatment, but this only after a rather exhaustive search. Mice receiving the vehicle only had normal kidney histology.

Pancreas. The pancreas appeared to be histologically normal in the control group as well as in both the CSA- and CSG-treated mice.

Behavioral and physical changes

Mice receiving CSA for a week or more became very aggressive and hyperactive; their hair stood up and acquired an "oil wet" form. Such changes were not observed in CSG-treated mice or in controls.

Discussion

A lot more is known about the pharmacodynamics and toxicity of CSA than CSG, both experimentally and clinically. CSA is well absorbed systemically and the relative distribution in different tissues has

been documented [1]. The drug accumulates in fairly high levels in the kidney, spleen, and progressively in the brain of mice; in our experiments, these are the tissues that appear to be damaged by CSA. Our toxicological studies indicate that with comparable blood levels of CSA and CSG and with the high blood levels of the two drugs, only CSA appears to have significant nephrotoxicity and to cause both weight loss and splenic atrophy. Whether this is due to differences in tissue penetration or simply to the single amino acid difference in molecular structure has yet to be established. Several studies have shown that, after oral administration, higher blood levels of CSG than of CSA are found in blood and serum of experimental animals [6, 7, 30] and man [15], suggesting that the single amino acid substitution probably results in increased absorption from the gastrointestinal tract and, perhaps, also in a different rate of metabolism of the drug.

In our mouse model, the increased nephrotoxicity of CSA compared to CSG has been documented biochemically [19]. Here we demonstrate in BALC/c mice treated with CSA in a dosage of 150 mg/kg, in excess of the immunotherapeutic range in mice, the occurrence of the typical histological changes in the kidney that have previously been described in man [20, 26], in rats [3, 9], and in primates [21]. Changes of proximal tubular vacuolation in animals treated with CSG were rare, which fits the observation that there was significantly less renal function impairment with CSG than with CSA (Table 1). We also did not observe any arteriolar change in the kidneys of mice treated with either CSA or CSG. This points to possible species differences in response to CSA, as vascular changes are observed in man but only rarely in experimental animals (e.g., in rats, they are observed only in spontaneously hypertensive rats [24]).

In the liver, we have been able to show mild to moderate histological changes with both drugs, although the histological changes appeared to be more marked in mice treated with CSA. There was also some functional impairment, at least in terms of serum bilirubin and albumin concentrations. The concentration of serum albumin was significantly lower in both the CSA- and CSG-treated animals than in controls; there was no difference in the concentration of albumin or bilirubin between the two experimental groups.

Currently, it is accepted that CSG is adequately immunosuppressive in the doses that were used in the pilot clinical study; the one troubling aspect of CSG in man is its claimed hepatotoxicity [15].

The marked weight loss in animals treated with CSA has been attributed, at least in part, to glycosuria [10, 11], something which was not observed in our study.

The reduction in body weight with CSA, but not with CSG, has been noted before in Sprague-Dawley rats [17]. It is probably compounded by reduced food intake.

CSA has, in other models, been shown to induce behavioral changes. In our model, animals treated with CSA showed marked hyperactivity and irritability, which would fit with the observed accumulation of CSA in the brain of mice [1]. The complete absence of these symptoms in mice treated with CSG in our study is of great interest and makes the assessment of central nervous system tissue levels of critical importance. In man, nervous system changes such as those of tremor and depression are important clinically; should CSG show a similar lack of neurotoxicity in man, this would be a major advantage of the drug - independent of other benefits - when compared to CSA.

Of all the changes observed in our model, the most intriguing is the observation of splenic atrophy with CSA but, again, not with CSG. The reduction in splenic weight was associated with marked and characteristic histological changes. The effect of CSA on lymphoid tissue has been mentioned before [23]. Also, it has been observed in rats that CSA can cause atrophy of the thymus [2]. We have observed gross histological changes in the spleens of mice treated with CSA. These changes appear to us to be due to actual cellular death. The changes appear to involve both the T and the B cell areas of the spleen, yet are more prominent in the T cell areas. We have not observed any vascular changes in the spleen; therefore, it is unlikely that the reduction in cellularity and the involutional changes are due to ischemia. The mechanism of these changes in the spleen and their potential significance, both in clinical terms and in terms of the mechanism of action of CSA in causing immunosuppression and graft acceptance, can only be conjectured at this stage. CSA has selective effects on different populations of lymphocytes in vitro [29]. Thus, current experiments are aimed at elucidating the exact proportions of mononuclear cells in the spleen that are sensitive to CSA.

Although it seems unlikely that patients given CSA develop splenic atrophy, this has not yet been specifically investigated. As the indications for using CSA increase, and particularly as CSA is now being used in younger age groups to treat, for example, juvenile onset diabetes mellitus [25], it would appear important to test for splenic size and function in patients who have been given relatively large doses for a long time. Splenic size is easy to mea-

sure and functional assessments may be helpful. The importance of this is obvious, as not only surgical splenectomy [14] but also functional asplenia is attended by a risk of overwhelming bacterial infection, particularly in children. This may assume even greater significance in the immunosuppressed individual.

Our toxicity study leads us to conclude that at least in the BALB/c mouse model, and with both drugs tested at levels in excess of the immunotherapeutic range, CSG appears to be a relatively safer drug than CSA, even when the crude but real parameter of total survival is considered. We feel that CSG requires more rigorous testing, both experimentally and clinically, since CSA is not completely without clinical problems.

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