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The influence of cyclosporin A on alloantibody responses in inbred rats: provisional evidence for a serum factor with antiidiotypic activity

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SUMMARY

The effect of cyclosporin A (CsA) on alloantibody synthesis has been investigated in inbred F344 ($RT1^{lvl}$) rats receiving weekly transfusions of DA ($RT1^{a}$) rat whole blood. Whereas repeated transfusion resulted in a persistent alloantibody response (Group I) administration of CsA (15 mg/kg/day) from either days 0–7 (Group II), days 8–49 (Group III) or days 15–49 (Group IV) resulted in the eventual suppression of alloantibody responses before the end of the experiment on day 49. Antiidiotypic activity was detected in sera obtained on day 49 from animals in Groups II, III and IV, shown to reside in the serum fraction of apparent molecular mass of between 150 and 170 kD and to be specific for alloantisera raised in F344 and the closely related LEW ($RT1^{l}$) rats but not the unrelated AO ($RT1^{u}$) strain. These experiments suggest that the immunosuppressive action of CsA may, in part, be due to the development of anti-idiotypic activity whose nature remains to be more fully characterized.

Keywords Antiidiotypic activity cyclosporine A blood transfusion

INTRODUCTION

Alloantibody responses have been of interest to clinicians involved in kidney transplantation for two principal reasons; firstly, alloantibodies to class I major histocompatibility complex (MHC) antigens may cause hyperacute rejection (Kissmeyer-Nielson *et al.*, 1966) and, secondly, it has been suggested that alloantibodies to class II MHC antigens enhance graft survival (Davies & Atkins, 1974; Soulillou *et al.*, 1976). Given the ability of the relatively new immunosuppressive agent cyclosporin A (CsA) to inhibit primary T dependent antibody responses (Lindsey *et al.*, 1980; Klaus & Dongworth, 1982), it is perhaps surprising that the effect of CsA on alloantibody responses has received so little attention. Only recently have clinicians made a systematic attempt to prevent alloantibody synthesis in patients receiving transfusions before transplantation (Hillis *et al.*, 1985).

As a result of this interest, and the paucity of studies concerned with the effect of CsA on alloantibody synthesis in experimental animals, we decided to investigate the influence of CsA on alloantibody responses in inbred rats receiving repeated transfusions. In addition, a resurgence of interest in the possible immunosuppressive role of antiidiotypic antibodies (Reed *et al.*, 1985; Sachs, Bluestone & Epstein, 1985) has prompted us to look for such antiidiotypic activity in transfused rats treated with CsA.

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MATERIALS AND METHODS

Animals

Male F344 ($RT1^{lvl}$, 200–250 g) and AO ($RT1^{u}$, 200–250 g) rats were obtained from Harlan Olac Ltd, Bicester, UK. Male DA ($RT1^{avl}$, 200–250 g) and LEW ($RT1^{l}$, 250–300 g) rats were obtained from Bantin and Kingman Ltd, Hull, UK. All animals were housed in a temperature controlled environment and received Oxoid pasteurized rat and mouse breeding diet with free access to tap water.

Cyclosporin A

Cyclosporin A (CsA) was obtained from Sandoz Ltd, Switzerland, in powder form and initially dissolved in anhydrous ethanol. A 15% (w/v) solution of CsA in 10% (v/v) ethanol in olive oil (Boots PLC, Nottingham, UK) was then prepared. This solution was administered to the conscious rat at a dose of 15 mg/kg/day by gastric intubation using a 4-fine gauge intravenous cannula (Portex Ltd, Hythe, UK).

Experimental protocol

Twenty Fischer rats were divided into four groups of five rats. On the first day of the experiment and at weekly intervals

thereafter each rat received an intravenous transfusion of 0·75 ml DA rat whole blood which had previously been collected into citrated tubes. In addition to this transfusion, rats in the second group (Group II) received CsA for the first 7 days of the experiment while those in Groups III and IV received CsA from day 8 and 15 respectively until the end of the experiment on day 49. Rats in Group I received no drug treatment. Blood samples from all animals were obtained from the tail vein under ether anaesthesia immediately before each transfusion. Sera expressed from clotted blood were heat inactivated at 56°C for 45 min and ultracentrifuged, to remove immune complexes, for 60 min at 100,000 *g* before being stored at −20°C.

Alloantibody assays

Complement-dependent lymphocytotoxicity. Complement-dependent lymphocytotoxicity (CDC) was performed by a standard technique (Burgos, French & Batchelor, 1974) using DA rat splenocytes as target cells.

Indirect haemagglutination assay. This assay was performed by a standard technique (Power, Cunningham & Catto, 1987) using DA rat erythrocytes as target cells and sera from a normal Fischer male as control.

Erythrocyte antibody rosette inhibition assay. This assay was performed by a standard technique (MacLeod *et al.*, 1982) using serum from the pretransfusion sample as the control and DA rat splenocytes as target cells.

Antiidiotypic antibody assays. Antiidiotypic antibody activity was detected by a modification of the short antiidiotypic antibody assay reported by Suciu-Foca *et al.* (1983). All putative antiidiotypic antisera possessed no detectable alloantibody activity by IHA, EAI or CDC assays and were obtained on day 49 of the experiment.

Hyperimmune serum was produced by two intraperitoneal injections of 15×10^6 DA splenocytes, each a fortnight apart. Pooled normal Fischer rat serum (NFRS; $n=8$) was used as a control. Antiidiotypic activity at each dilution was expressed as percentage inhibition of CDC and was calculated as follows.

$$\% \text{ Inhibition of CDC} = 100 \times \frac{Sc - St}{Sc}$$

where Sc = CDC activity of hyperimmune serum incubated with NFRS and St = CDC activity of hyperimmune serum incubated with test serum. Results were expressed as the last dilution of test sera ($-\log_2$) to inhibit CDC by more than 30%.

Serum fractionation

Two millilitres of pooled day 0 or day 49 sera from groups II, III and IV were fractionated on a 2·5 × 60 cm Sephadex G200 column (Pharmacia, Uppsala, Sweden). Fractions were eluted with PBS pH 7·3. The column was calibrated with the following standards: B-amylase (Sigma Chemical Co. Ltd, Poole, UK), alcohol dehydrogenase (Sigma), human serum albumin (Hoescht UK Ltd, Hounslow, UK), ovalbumin (Koch Light Ltd, Haverhill, UK) and carbonic anhydrase (Sigma) and the void volume determined using blue dextran (Sigma). Three major peaks were obtained. The middle peak contained IgG which was concentrated to 15 mg/ml; the other two peaks were pooled and concentrated to the same volume.

Determination of serum IgG and IgM alloantibody activity

Twenty-five microlitres of test sera were assayed at a dilution of 1:2 in a 96-well microtitre plate which had been previously blocked with 200 μl of 1% BSA in PBS. Twenty-five microlitres of DA red cells at 200×10^6/ml were added to each well. The plates were then incubated at 22°C for 1 h after which they were washed three times with 1% BSA in PBS. Twenty-five microlitres of either biotinylated goat anti-rat IgM (Cambridge Bioscience, Cambridge, England) or goat anti-rat IgG (Stratech Scientific Ltd, London, England), biotinylated by the method of Smith *et al.* (1986) were added at dilutions of 1:250 and 1:800 respectively, and the plates incubated for another hour at room temperature. Plates were then washed a further three times in 1% BSA, before 25 μl of a 1:50 dilution of ^{125}I-streptavidin (Amersham) was added to each well. After incubation at room temperature for 30 min, plates were washed four times with 1% BSA in PBS before cells from each well were harvested into microtubes and activity measured using a gamma-counter. Results were expressed as % binding using the following formula:

$$\% \text{ binding} = 100 \times \frac{T - B}{M - B}$$

where M is maximal activity present in 25 μl of a 1:50 dilution of ^{125}I-streptavidin, B is the background activity measured in the absence of test sera and T is the activity obtained in the presence of a test serum. Specificity of these heavy chain specific antisera was established using monoclonal rat IgG and IgM anti-RT1A^a alloantibodies.

RESULTS

Alloantibody activity

CDC. The cytotoxic activity of control and CsA treated Fischer rats in response to weekly whole DA blood transfusions is shown in Fig. 1. In all four groups this response was maximal 7 days after the initial transfusion but was significantly diminished by day 14. After this time the mean cytotoxic activity in Group I (Fig. 1a) fell further until day 28 from which point until the end of the experiment on day 49 the mean titre remained around 1/16. In contrast, by day 14, 7 days after the end of CsA treatment, sera from three of the five rats in Group II (Fig. 1b) had no cytotoxic activity and by day 21 this activity was abolished in all five rats. On day 21, 14 days after starting the administration of CsA to rats in Group III (Fig. 1c), sera from two of the five animals had no cytotoxic activity. By day 35 the CDC titres of all five rats in this group had returned to zero. Administration of CsA from day 14 resulted in the abolition of cytotoxic antibody in one of the five rats in group IV (Fig. 1d) by day 21 and in all of the rats by day 28.

IHA. Before the start of the experiment one rat in Group I, two rats in Group II and one rat in Group IV had weak pre-existing haemagglutinating antibody activity (Fig. 2). Naturally occurring anti-RT1.A antibodies have previously been described by Gunther and colleagues who suggested that they occurred in response to either polyclonal stimulation by bacterial mitogens, environmental antigens or were a result of the spontaneous 'background' of the immune system (Gunther, Elleser-Beile & Hedrich, 1983).

As with CDC, a peak in mean IHA activity occurred on day 7 in all four groups. On day 14 the mean IHA titre in Group I

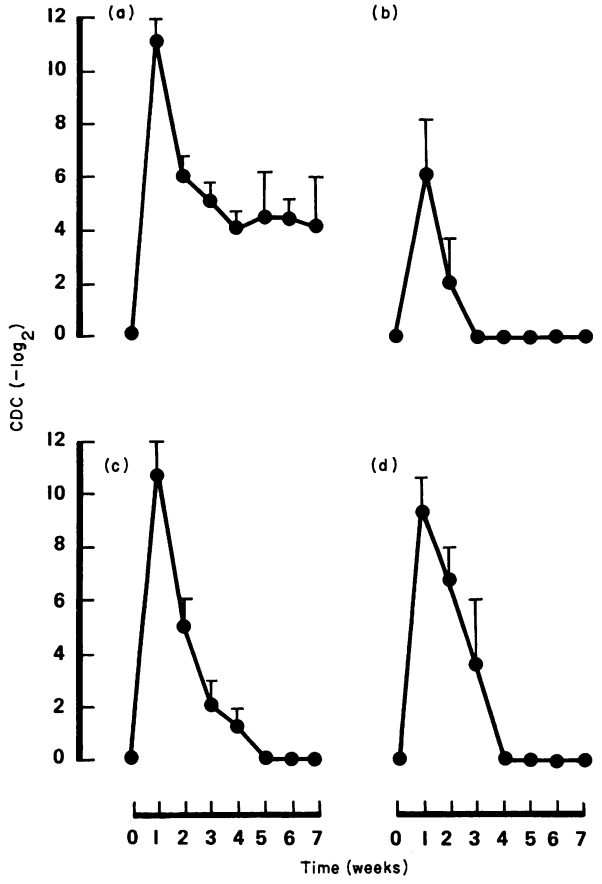

Fig. 1. Alloantibody responses detected by CDC in Fischer rats transfused weekly with DA blood: (a) Group I received no additional therapy; (b) Group II CsA (days 0–7); (c) Group III CsA (days 8–49); and (d) Group IV CsA (days 15–49). Results expressed as mean + 1 s.d.

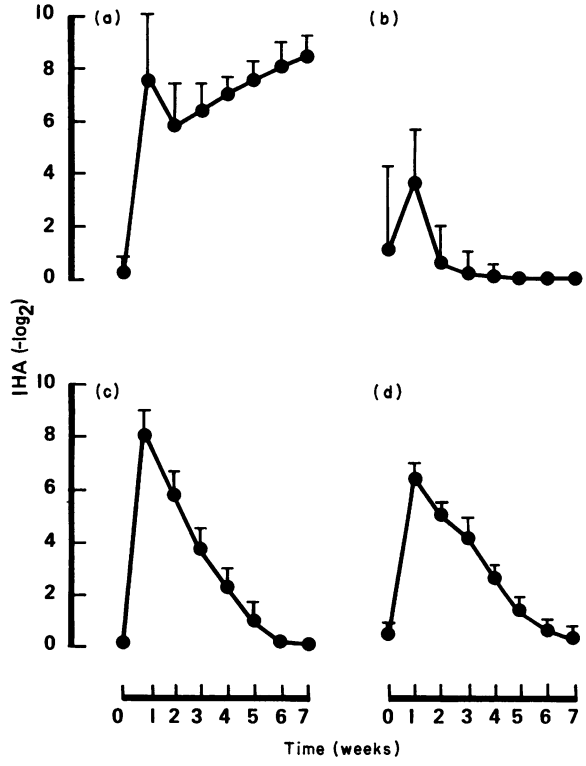

Fig. 2. Alloantibody responses detected by IHA in Fischer rats transfused weekly with DA blood: (a) Group I received no additional therapy; (b) Group II CsA (days 0–7); (c) Group III CsA (days 8–49); and (d) Group IV CsA (days 15–49). Results expressed as mean + 1 s.d.

had fallen from its level on day 7 but the mean titre rose continuously thereafter until the end of the experiment (Fig. 2a). The day 7 peak in IHA activity in rats treated with CsA from days 0–7 was significantly lower than that in other groups (Fig. 2b). Of the two rats in Group II with pre-existing antibody, the activity detected by IHA in one had disappeared by day 14 but persisted in the other until day 28; by this time the remaining four rats had no measurable antibody titre. Only at day 35 was the IHA activity of all five rats zero. A very similar pattern of IHA activity was observed with rats in Groups III (Fig. 2c) and IV (Fig. 2d). From the maximal IHA response at day 7 the mean haemagglutinating activity fell steadily in both groups. By day 42 none of the rats in Group III had any haemagglutinating antibody activity; however, activity remained in one rat in Group IV until the end of the experiment.

EAI. In contrast to the mean level of EAI in Group I (Fig. 3a), which rose almost continuously throughout the experiment, the level of EAI in Group II did not become significant (> 20%) at any time (Fig. 3b). Seven days after the initial transfusion, the mean EAI level in Group III was 40% and this activity was maintained until day 14 (Fig. 3c) when it fell steadily to zero on day 35. In Group IV, EAI activity decreased from the introduction of CsA treatment on day 14 until the end of the experiment; the mean activity fell below 20% on day 35 (Fig. 3d).

Antiidiotypic activity. Sera, obtained on day 49 and pooled from rats in Group II, III or IV contained antiidiotypic activity against Fischer anti-DA and Lewis anti-DA hyperimmune alloantisera but not against AO anti-DA alloantisera (Table 1). In contrast, pooled sera obtained on day 0 contained no antiidiotypic antibody activity when tested against Fischer, Lewis or AO anti-DA alloantisera. The one animal in Group I which spontaneously lost cytotoxic alloantibody activity did not possess detectable antiidiotypic activity.

Fractionation of pooled day 49 sera from Groups II, III and IV by G200 Sephadex column chromatography showed that antiidiotypic activity was present only in peak 2 which contained material of apparent molecular mass 150–180 kD (Table 2). This activity was again directed against hyperimmune alloantibodies raised in Fischer and Lewis rats.

Serum IgM and IgG alloantibody levels

The serum IgM and IgG alloantibody levels against DA erythrocytes of each of the four groups over the first 28 days of the experiment are shown in Fig. 4. Weekly whole DA blood transfusions to otherwise untreated Fischer rats resulted in a strong IgM response over the first 7 days of the study which persisted until day 14 (Fig. 4a). Between days 14 and 21, however, the level of IgM alloantibodies fell. This pattern was repeated in rats treated with CsA from either day 7 or day 14 until the end of the study (Fig. 4c and d). Although the animals in Group II, who received CsA for the first 7 days of the experiment, had a level of IgM alloantibodies at day 7 similar to

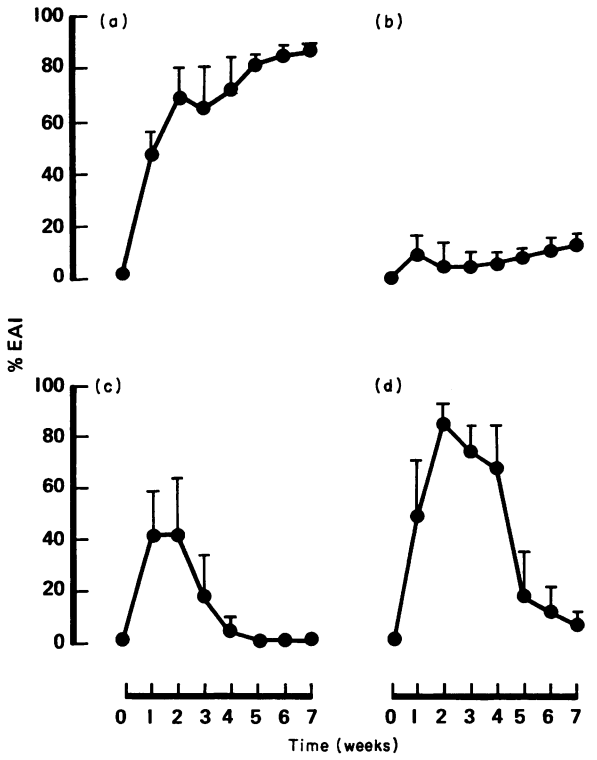

Fig. 3. Alloantibody responses detected by EAI in Fischer rats transfused weekly with DA blood: (a) Group I received no additional therapy; (b) Group II CsA (days 0–7); (c) Group III CsA (days 8–49); and (d) Group IV CsA (days 15–49). Results expressed as mean + 1 s.d.

Table 1. Antiidiotypic activity and specificity in day 49 serum samples

	Hyperimmune alloantisera		
Group	Fischer anti-DA	Lewis anti-DA	AO anti-DA
II	4·0+1·2	2·3+0·5	0+0
III	4·0+1·6	1·4+1·3	0+0
IV	2·0+0	3·0+1·7	0+0

Results are expressed as the last dilution of test sera ($-\log_2$) to inhibit CDC by more than 30% (mean + 1 s.d.)

that of the other groups (Fig. 4b), these levels had fallen by day 14.

Whereas IgG alloantibodies were first detected in Group I on day 7 and increased throughout the first 28 days of the experiment (Fig. 4a), the administration of CsA for the initial 7 days (Group II) resulted in no detectable IgG response (Fig. 4b). Administration of CsA from either day 7 or 14 (Groups III and IV) resulted in a reduction in IgG levels from day 14 until day 28 (Fig. 4c and d) and a markedly different pattern of IgG response to that observed in Group I.

DISCUSSION

This study confirms the ability of cyclosporin A to abrogate the alloantibody response of inbred rats to repeated blood transfusion from a single strain. The effect occurred in all animals given CsA whether it was administered for the first week alone or from the beginning of the second or third weeks to the end of the study. Moreover, antiidiotypic antibody activity developed in all animals that received both CsA and blood transfusions.

Animals not given CsA (Group I) did not normally lose alloantibody activity as assessed by any of the alloantibody assays. These results differ from those reported by Fabre & Morris (1972), who found that twice weekly transfusions of 0·5

Table 2. Antiidiotypic activity in fractionated pooled day 49 serum samples

	Hyperimmune alloantisera			
	Fischer anti-DA		Lewis anti-DA	
Group	Peak 2	Peaks 1+3	peak 1	Peaks 2+3
II*	3	0	2	0
III*	2	0	2	0
IV*	0	0	0	0
IV†	1	0	0	0

Results represent the titre obtained for each fraction of pooled sera and are expressed as the last dilution of test sera ($-\log_2$) to inhibit CDC by more than 30%.
* IgG concentration = 15 mg/ml.
† IgG concentration = 30 mg/ml.

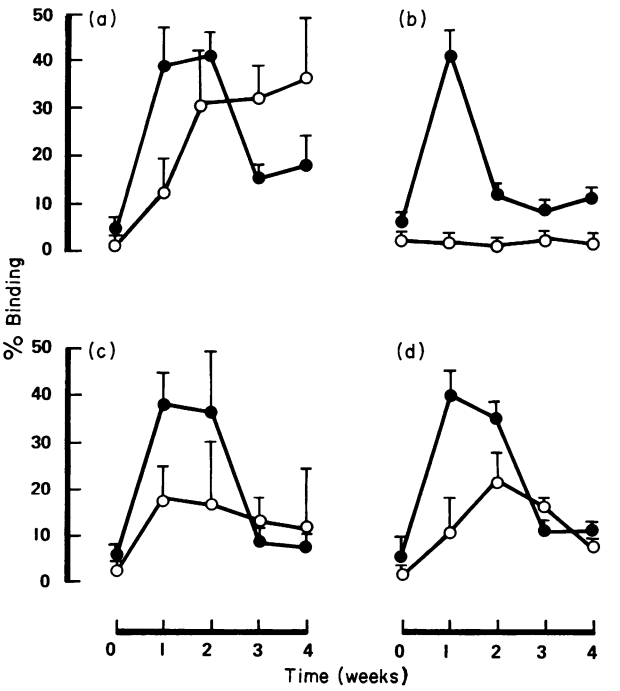

Fig. 4. Serum IgM (●) and IgG (○) alloantibody binding in Fischer rats transfused weekly with DA blood detected by radioimmunoassay: (a) Group I received no additional therapy; (b) group II CsA (days 0–7); (c) Group III CsA (days 8–49); and (d) Group IV CsA (days 15–49). Results expressed as mean + 1 s.d.

ml DA blood into 10 Lewis rats resulted in greatly diminished cytotoxic alloantibody activity at day 28 after an initial peak at day 7. In that study seven DA rats received twice weekly transfusions of 0·5 ml Lewis blood. None of these rats had detectable cytotoxic alloantibody activity at day 14. The data reported here are, however, probably compatible with the study of Lenhard *et al.* (1985), who found that as many as fifteen 1 ml transfusions were required to reduce or abolish the cytotoxic alloantibody response using Brown Norway rats as recipients of Lewis transfusions.

Cyclosporin A is known to suppress primary antibody responses to T dependent antigens (Lindsey *et al.*, 1980; Klaus & Dongworth, 1982). There have been few systematic studies of its effect on alloantibody formation, although Homan *et al.* (1980) have previously reported that CsA abolished the cytotoxic alloantibody response by day 7 in four of five Lewis rats grafted with DA kidneys and given CsA 10 mg/kg/day by mouth for 14 days. It was of interest, therefore, to find in our study that antibody formation occurred in animals given CsA during the first week of the protocol and that this activity declined despite further transfusion and cessation of CsA. Binding studies using ^{125}I- streptavidin indicated that the activity detected by our three alloantibody assays in rats from group II was due to IgM rather than IgG antibodies. Previous experiments using a variety of antigens (Lindsey *et al.*, 1980; Klaus & Dongworth, 1982) did not suggest that CsA would have any effect on established alloantibody responses. The gradual decline in alloantibody levels seen in animals immunized by one or two transfusions and then given CsA with repeated transfusions (Groups III and IV respectively), was, therefore, unexpected. In our initial studies, we considered that CsA must have prevented the immunoglobulin heavy chain class switch in B lymphocytes, a process which requires T cell help (Davie & Paul, 1974). Binding studies showed, however, that IgG production had already occurred at day 7 and was well established by day 14 which suggests that IgG synthesis was suppressed by CsA; whether this phenomenon is mediated through a direct effect of CsA on B lymphocytes or indirectly via the selective sparing of suppressor T lymphocytes is not known. We have previously reported that the administration of CsA over an 8 week period has little effect on established alloantibody responses produced in Lewis rats by intraperitoneal injection of DA splenocytes (Power *et al.*, 1987). The present study, therefore, suggests that either alloantibody responses produced by blood transfusion differ fundamentally from those elicited by other methods or that the early IgG alloantibody response, up to 14 days after stimulation, can be suppressed but loses this characteristic as it matures. Further studies will be required to differentiate these two possibilities.

Antiidiotypic activity has not been previously reported in experimental animals given CsA. Persistence of antigen has, however, been detected in rabbits given BSA. The presence of soluble antigen might therefore appear to be the simplest explanation for the presence of antiidiotypic activity in sera from animals given CsA and repeated allogeneic blood transfusions. In the present study, however, there was a degree of specificity in the antibody activity which was unexpected. These sera inhibited the cytotoxic activity of sera raised in the closely related F344 and LEW strains but not AO. As column chromatography indicated that the apparent molecular mass of this soluble factor lies between 150 and 180 kD it could be either a modified transplantation antigen or, more likely, an IgG antibody. In summary, these experiments show that CsA is capable of inhibiting IgG alloantibody responses and suggest that part of the immunosuppressive action of CsA may be due to the development of a soluble factor with antiidiotypic activity. Further studies will be necessary, however, to determine the biochemical nature of this factor and its role in the modification of alloantibody responses both *in vitro* and *in vivo*.

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