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Prolongation of life in female NZB/NZW (F_1) hybrid mice by cyclosporin A

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SUMMARY

Oral administration of cyclosporin A (Cy A), an immunosuppressive agent, prolonged the life span of female NZB/NZW (F_1) hybrid mice (NZB/W). The most prominent feature of this study was the reduction of glomerular proliferation, proteinurea, and delay of renal failure. Protection from renal damage, in treated mice, occurred despite similar degrees of perivascular cellular infiltration in the kidney and other organs, and glomerular deposition of immunoglobulin and complement. Treatment did not influence the hypergammaglobulinaemia, high levels of circulating immune complexes and later development of autoimmune haemolytic anaemia typically associated with this murine disease. The levels of antibodies to double stranded DNA (dsDNA), an important disease marker, and the response to sheep red blood cells were reduced in the Cy A treated mice, in contrast with the untreated mice. Levels of rheumatoid factors were increased in Cy A treated mice, when compared with untreated mice, and this could play a role in prolongation of life associated with protection from glomerular damage, although it was considered that inhibitory effects on T cell function were a more likely mechanism.

Keywords cyclosporin A protection NZB/NZW mice

INTRODUCTION

Cyclosporin A (Cy A), an immunosuppressive agent used in experimental and clinical transplantation, has also been found to have efficacy in experimental allergic encephalomyelitis (Bolton *et al.*, 1982) and adjuvant arthritis (Borel, Wiesinger & Gubler, 1978) in rats. There is also evidence that it can ameliorate spontaneous early onset diabetes mellitus (Laupacis *et al.*, 1983) and induced anterior uveitis (Nussenblatt *et al.*, 1981) in rats. Unlike other immunosuppressive agents, Cy A is not significantly toxic to bone marrow or gut epithelium at dose levels which can inhibit immune responses and it has been considered as a possible therapeutic agent for spontaneous conditions in man associated with autoimmunity. For example Cy A treatment improved liver function in some patients with primary biliary cirrhosis (Routhier *et al.*, 1980).

The NZB × NZW F_1 hybrid mouse (NZB/W) provides a model of spontaneous immunoproliferative disease with similarities to human systemic lupus erythematosus (SLE). The female NZB/W typically develops antibodies to double stranded DNA (dsDNA) and subsequent glomerulonephritis becoming clinically apparent around 6 months of age. This murine syndrome thus provides an excellent experimental model to test the efficacy of Cy A. The present paper describes the effects of continuous oral treatment of female NZB/W mice with Cy A, with respect to longevity and development of autoimmunity.

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MATERIALS AND METHODS

Animals. NZB/NZW (F_1) hybrid mice reared at the Kennedy Institute were used at 2 months of age. The mice were fed on a high protein diet, D.M.D. (Labshore). Mice were randomly selected from a litter for Cy A and control treatment. The mice were subsequently kept in separate cages.

Treatment. Cy A was a gift from Dr J. Borel, Sandoz, Basle, Switzerland. It was dissolved in olive oil by stirring for 30 min, and given immediately at an immunosuppressive dose of 100 mg/kg, five times a week, using a blunt ended curved needle and syringe. Treatment commenced at 2 months of age and was continued until death.

Histology. Mice (three to six) were killed at various times during the course of treatment, and spleens, thymus and mesenteric lymph nodes were weighed. These tissues in addition to one kidney, liver, lung and heart were prepared for routine histological examination. The other kidney was embedded in Tissue-Tek II O.C.T. (Raymond A. Lamb) and frozen in liquid nitrogen.

Direct immunofluorescence. Cryostat sections of frozen kidney were incubated with fluoresceinated antiserum specific for mouse IgM, IgG and C3 (Nordic). A working dilution of 1:10 was used for an incubation period of 30 min. After washing in phosphate-buffered saline (PBS), tissues were examined in a microscope equipped with an u.v. light source.

Proteinurea. Proteinurea was assessed by staining with bromophenol blue (Knight, Adams & Purves, 1977). Proteinurea values above 0·3 g/100 ml were considered above normal in these mice (Harris & Chandler, 1981).

Haematology. Blood was collected into potassium ethylendiamine tetra-acetic acid (EDTA) tubes. Haemoglobin levels were measured by the cyan methaemoglobin method, and estimated spectrophotometrically. Reticulocytes were estimated as a percentage of at least 200 mature RBC in smears prepared from peripheral blood and stained with Cresyl blue, in 'ready to use' Merret tubes (Almedica AG, Basle, Switzerland).

Anti-erythrocyte antibody (anti-globulin test). Direct testing was done against washed (three times) mouse red cells using rabbit anti-mouse immunoglobulin antiserum. From comparison with tests using red cells from young, normal mice (C57Bl/6, CBA), dilutions of 1:80 and above were assumed to be positive. Microtitre plates were used, each containing 20 μl 5% red blood cells and 100 μl antiserum, results being read after 1 h at 37°C and several hours at 4°C.

Serum immunoglobulin levels. Serum IgM and IgG levels were determined using the single radial immunodiffusion method (Mancini, Carbonara & Heremans, 1965). Rabbit anti-mouse IgM and IgG anti-serum were obtained from Nordic. The IgM plates were allowed to equilibrate for 72 h and IgG for 48 h before the ring was directly measured.

Anti-ds DNA antibodies. One hundred microlitres of a 1% aqueous solution of protamine sulphate (Grade II, Sigma) was incubated in a 96 well microtitre plate (Nunc) for 1 h at 37°C. The plates were then washed three times with PBS. One hundred microlitres of 15 μg/ml mouse DNA (Marmur, 1961) in PBS, was then incubated for 1 h at 37°C. The plates were washed three times with PBS. Any free sites remaining on the plates were blocked with 0·4% bovine serum albumin (BSA)-PBS for 1 h at 37°C. The plates were again washed three times with PBS. One hundred microlitres of a 1:100 dilution of mouse serum in PBS, 0·4% BSA, 0·05% Tween 20 (PBS, BSA, Tween), with doubling dilutions to 1:12,800 was then added to the plates for 1 h at 37°C. The plates were washed three times with PBS Tween, and three times with PBS. One hundred microlitres of 1:1,000 dilution in PBS, BSA, Tween alkaline phosphatase conjugated rabbit anti-mouse IgG (Sigma) was added to the plates and incubated for 1 h at 37°C. The plates were washed three times in PBS-Tween and three times in PBS. Finally, 100 μl of the alkaline phosphatase substrate, *p*-nitrophenyl phosphate (Sigma) was added at 5 mg/ml, and after 30 min incubation at 37°C the reaction stopped by the addition of 100 μl 2 M NaOH. Hydrolysis of the substrate *p*-nitrophenylate was determined by measuring the absorbance at 410 nm on an ELISA reader (Titretik, Flow).

Anti-single stranded (ss)DNA antibodies. ssDNA was prepared by boiling the mouse DNA for 15 min, plunging it into ice, and immediately coating the microtitre plates with 100 μl 15 μg/ml ssDNA. Antibodies to ssDNA were measured as for those to dsDNA.

Immune complex assay. Rabbit C1q was prepared according to the method of Yonemasu &

Stroud (1971). One hundred microlitres of rabbit C1q (10 μg/ml) in PBS was incubated in a 96 well microtitre plate (Nunc) for 3 days at 4°C. The plates were then washed three times with PBS and any free sites remaining were blocked for 2 h at room temperature with 100 μl 0·01% wt/vol. gelatin solution in PBS. Meanwhile, 5 μl serum was incubated with 10 μl 0·2 M EDTA, pH 7·5 for 30 min at 37°C and transferred to an ice bath where a further 35 μl 0·2 M EDTA, pH 7·5 was added to make a 1:10 dilution. Doubling dilutions of the serum were added to the blocked C1q coated plates and any IgG bound to the C1q detected with an alkaline phosphatase conjugated rabbit anti-mouse IgG (Sigma) as in the dsDNA antibody assay.

Anti-globulin antibodies. Human IgG(10 μg/ml) (Miles) was used to coat 96 well microtitre plates, and any free sites remaining were blocked with 0·4% BSA in PBS. Doubling dilutions of the serum was added to the blocked human IgG plates and any bound mouse IgG was detected with an alkaline phosphatase conjugated rabbit anti-mouse IgG (Sigma) as in the dsDNA antibody assay.

Response to sheep red cells (SRC). Using the Cunningham chamber method (Cunningham, Smith & Mercer, 1966), direct plaque forming cells were enumerated in splenic cell suspensions on day 4 following the i.p. injection of 2×10^8 SRC.

Statistics. Chi square distribution was used to determine the statistical significance of the results.

RESULTS

The results of Fig. 1 show that Cy A, given orally at a daily dose of 100 mg/kg body weight significantly prolonged the life span of female NZB/W mice. Immunosuppressive treatment was started in these mice at 2 months of age, before clinical or significant serological evidence of autoimmunity had developed. From the survival curves, which indicated the cumulative deaths in treated and untreated mice, it was found that Cy A significantly prolonged the 50% mortality period which was around 11 months in untreated animals ($\chi^2 = 12{\cdot}89$, $P < 0{\cdot}0005$). The number of Cy A treated mice still alive at 21 months was highly significantly different from control, untreated mice ($\chi 2 = 14{\cdot}5$, $P < 0{\cdot}0005$).

Treatment with Cy A did not significantly influence the body weights or the cellularity of the peripheral lymphoid tissues. Splenomegaly and increase of mesenteric lymph node weight occurred in both treated and untreated mice. There was no significant effect on thymic tissue commensurate with the clinical status of individual mice but, treatment with Cy A preserved the thymic cortex from cellular loss. This could perhaps be related to the continued well being of the mice whose life span was prolonged by treatment with Cy A.

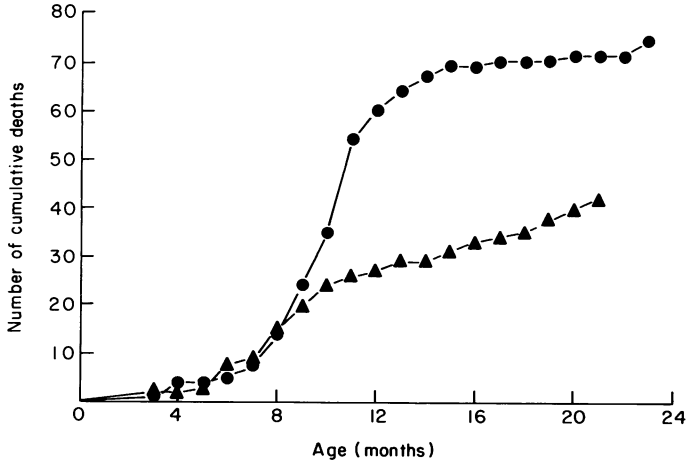

Fig. 1. The effect of continuous oral Cy A (▲) on the survival of female NZB/W F_1 hybrid mice. Untreated = ●.

Table 1. Histological assessment of perivascular infiltration of tissues in untreated female NZB/W mice

Age (months)	Kidney		Liver		Lung		Thymus		Salivary glands	
	C	Cy	C	Cy	C	Cy	C	Cy	C	Cy
4	−	−	−	−	−	−	W	W	−	−
5	±	−	−	−	±	−	W	W	−	−
6	−	+	−	−	−	+	W	W	−	−
7	++	+	±	−	+	−	W	W	−	−
8	++	±	±	±	++	+	A	W	−	−
9–10	+++	++	+	+	+++	+	A	W	+	−
11–13	++++	++++	±	±	++++	+++	A	A	++	+

C = untreated female NZB/W mice.
Cy = Cy A treated female NZB/W mice (100 mg/kg).
A = thymus cortex atrophied.
W = thymus cortex well preserved.
Degree of perivascular infiltration: ± slight around blood vessel; + moderate around blood vessel; ++ heavy around blood vessel; +++ heavy around blood vessel & slight in tissue; ++++ dense around blood vessel & in tissue.

As shown in Table 1, histological examination revealed that Cy A did not influence the perivascular infiltration of tissues by lymphoid, plasma and phagocytic cells, characteristic of this murine syndrome (Harris & Chandler, 1981). Further to this it was found, as indicated in Table 2, that glomerular deposition of both immunoglobulin and complement was similar in both untreated and treated mice. Thus Cy A prevented damage to the glomerular endothelium of NZB/W mice by some means other than interfering with the deposition of immune complexes. Serial histological examination showed that the typical signs of glomerular damage such as increase of inflammatory cells with subsequent atrophy and fibrosis were delayed by Cy A treatment and this would appear to

Table 2. Deposition of immunoglobulin and complement in glomerulus and incidence of proteinurea in untreated and treated female NZB/W mice

Age (months)	IgM		IgG		Complement		Proteinurea >0·3 g/100 ml		
	C	Cy	C	Cy	C	Cy	C	Cy	
5	+	±	++	+	+	±	9/14	0/12	$\chi^2 = 11{\cdot}79$ $P < 0{\cdot}001$
7	+	+	+	+	+	+	9/11	10/18	$\chi^2 = 2{\cdot}08$ $P < 0{\cdot}2$
10	+	+	+++	++	+++	++	6/7	9/14	$\chi^2 = 1{\cdot}05$ $P < 0{\cdot}2$

C = untreated female NZB/W mice.
Cy = Cy A treated female NZB/W mice (100 mg/kg).
Proteinurea results represent number of mice with proteinurea 0·3 g/100 ml/total examined.
± faint staining of glomeruli; + light but definite staining of glomeruli; ++ moderate staining of glomeruli; +++ heavy staining of glomeruli.

Table 3. Haematological status and serum levels of IgM and IgG in control of Cy A treated female NZB/W mice (Cy)

				Serum immunoglobulin	
	Hb (g/dl)	Reticulocytes	Direct anti-globulin test	IgM (ring diam. mm)	IgG (mg/ml)
Control (> 10 months old)	9·6 ± 2·1	13·5 ± 14·8	17/17	11·6 ± 0·6	14·8 ± 0·5
Cy (10 months old)	10·1 ± 2·1	8·2 ± 13·2	28/28	13·5 ± 1·41	15·3 ± 0·6

The blood from 17 control mice and 28 treated mice (Cy) were examined. In view of the higher death rate in untreated mice, the control group providing data were a pool of survivors > 10 months old. The direct antiglobulin test represents the proportion of mice whose red cells were agglutinated by a dilution of the anti-globulin reagent > 1/80.

be the major reason for prolonged life span. Confirmation of this protection from renal damage, the usual cause of early deaths in female NZB/W mice was confirmed by the delayed onset of proteinuria. It was previously found (Harris & Chandler 1981) that normal glomerular filtration rates (GFR) persisted until late in untreated mice, even in the presence of marked proteinuria. Measurements of GFR were also normal in Cy A treated mice and are omitted.

Serial studies of treated and untreated female NZB/W female mice indicated no significant preventive effects of Cy A on the development of autoimmune haemolytic anaemia or of raised serum immunoglobulin levels, despite using an immunosuppressive dose. As seen from the results of Table 3, both treated and untreated mice were Coombs (anti-globulin) positive, anaemic, and showed a varying reticulocytosis, typical of murine autoimmune haemolytic anaemia, by 10 months of age. Serum IgM levels did not alter significantly in either treated or untreated female NZB/W mice during the period studied (2–10 months), but the IgG levels in both groups, as shown in Table 4, had more than doubled as compared to the levels in these mice at 4 months of age (6·8 ± 1·6 mg/ml).

In view of the renal findings of Table 2, circulating immune complexes were measured. Treatment with Cy A made no significant difference to the levels of circulating immune complexes (results omitted), and this coincided with the finding of glomerular deposits of immunoglobulin and

Table 4. Number of plaque forming cells/10^6 spleen cells in treated and untreated NZB/W mice

Age	Control	Pyritinol	Cy A
30 weeks	184 ± 333 (n = 12)	374 ± 304 (n = 12)	20 ± 16 (n = 12)

NZB/W mice were treated as indicated from 8 weeks of age and the number of direct plaque forming cells/10^6 spleen cells were measured on day 4 following i.p. injection of 2×10^8 sheep red blood cells in untreated and treated female NZB/W F_1 hybrid mice.

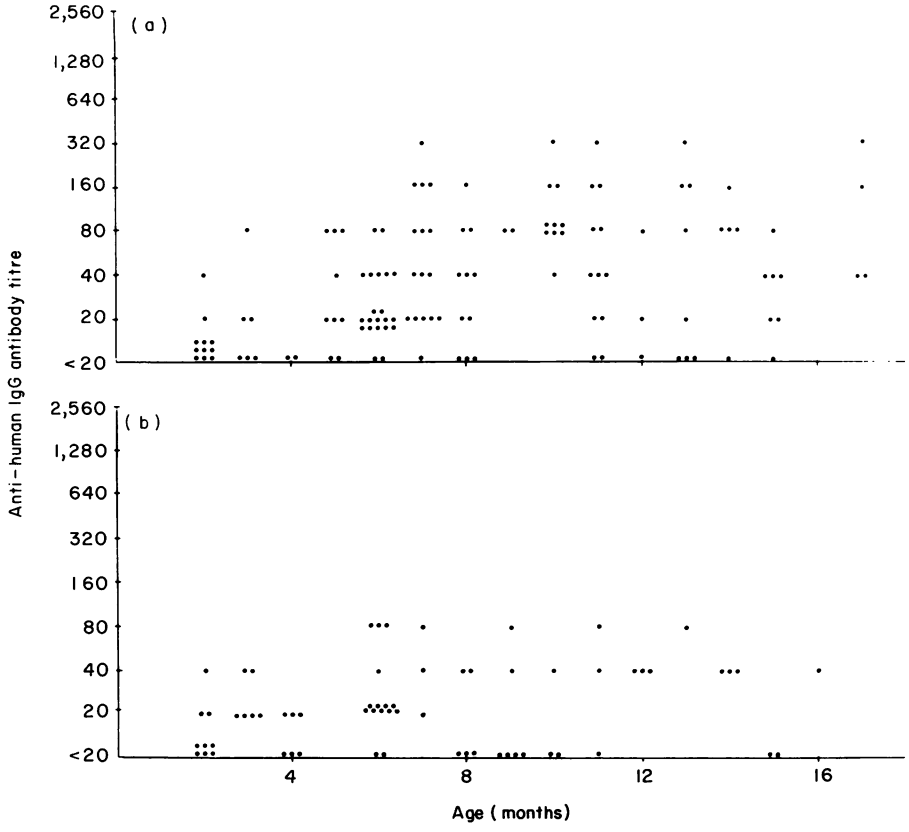

Fig. 2. Serum levels of anti-human IgG antibody in female NZB/W mice, comparing controls (b) with animals treated with oral Cy A (a) given as indicated in the text.

complement despite preservation of renal glomerular structure and function during treatment with Cy A.

Antibodies binding with human IgG, which closely resemble rheumatoid factors, were found in both control and Cy A treated female NZB/W mice, as shown in Fig. 2. Serial studies showed the titres of rheumatoid factors began to increase after 4 months of age and that this change was significantly more marked in Cy A treated mice as compared to untreated controls of similar ages ($\chi^2 = 8{\cdot}68$, $P < 0{\cdot}0005$). This difference was apparent by 8 months of age, and thus was not simply a feature of prolonged survival due to treatment.

Serial studies of antibodies to dsDNA are depicted in Fig. 3. Treatment with Cy A did not prevent the appearance of these antibodies. Comparisons in older mice were limited by the small numbers of surviving controls, but Cy A treatment may have resulted in some reduction of the levels of anti-dsDNA in animals >8 months old as compared to controls of the same age ($\chi^2 = 29{\cdot}93$, $P < 0{\cdot}0005$). However a progressive increase of anti-dsDNA did occur in mice treated with Cy A. Similar levels of antibodies to ssDNA were present in the serum of both treated and untreated mice without any significant difference in their levels and these results have been omitted.

Testing the responses of female NZB/W mice to SRC, a T cell-dependent antigen, was used as an indicator of the immunosuppressive effect of Cy A on normal immune responses. As shown in Table 4, Cy A, given for at least 20 weeks to female NZB/W mice, suppressed their response at day 4 to i.p. SRC, despite its lack of effect on the humoral autoimmune status of these mice, then or at later times. The responsiveness of female NZB/W mice, given pyritinol, a thiol compound which can also protect these mice (Harris & Chandler, 1981), is indicated for comparison.

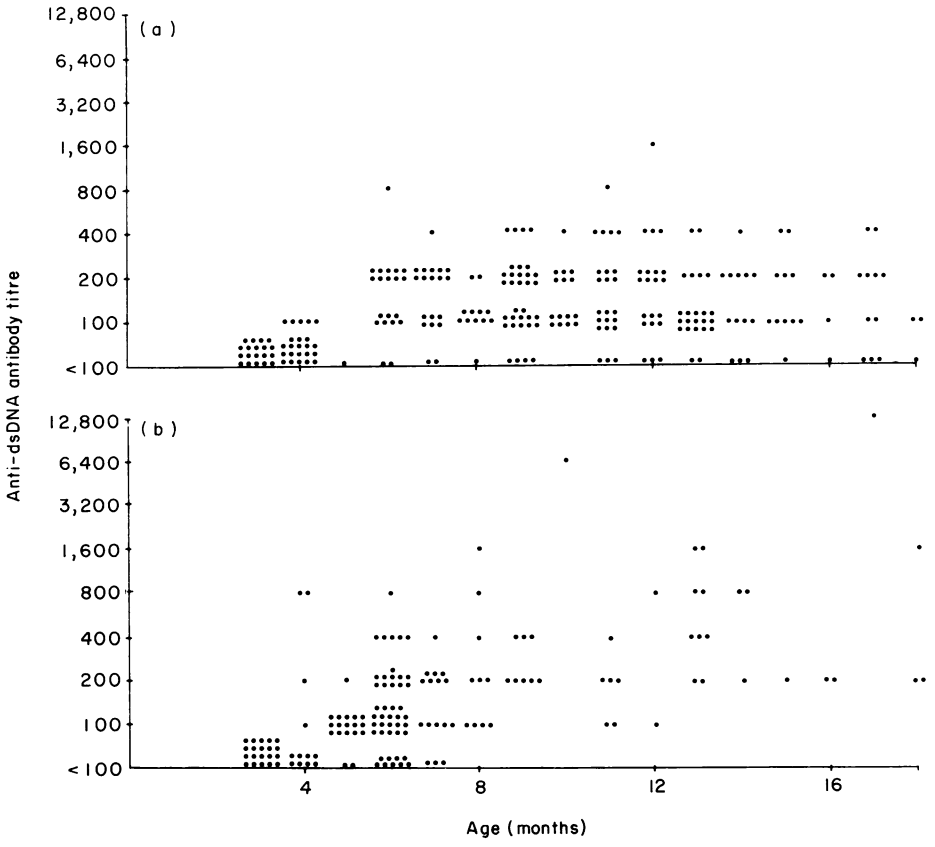

Fig. 3. Serial estimation of antibodies to dsDNA in (b) control female NZB/W mice and (a) animals treated with oral Cy A.

DISCUSSION

The female NZB/W F_1 hybrid mice used here typically developed an autoimmune glomerulonephritis, associated with increased levels of antibodies to dsDNA in their serum, which became clinically apparent with deaths beginning after 6 months of age, and with a 50% mortality around 11 months. Perivascular immunoproliferative changes in the kidneys and other organs are associated with the renal disease, but a previous study of the protective effects of oral pyrithioxine or pyritinol (Harris & Chandler, 1981), a compound with thiol groups, showed that the resulting prolonged life span of NZB/W female mice was associated with prevention of glomerular damage and preservation of renal function, but the typical perivascular infiltration by lymphocytes and plasma cells was not affected.

Prolongation of the life span of female NZB/W mice has been produced by dietary manipulation (Fernades, Yunis & Good, 1976), ionising radiation (Kotzin & Strober, 1979) and various other methods which damage the immune system (e.g. Gelfand & Steinberg, 1972). The present results show that Cy A, given at a non-toxic oral dose continuously over many weeks, was immunosuppressive and significantly prolonged the life span of these mice. CBA and C57Bl/6 mice which are not prone to develop spontaneous autoimmune renal disease can be given similar dosage of Cy A over lengthy periods of time without renal damage (personal observation).

The effects noted were strikingly similar to the protective action of penicillamine (Harris & Hutchins, 1979) and pyrithioxine (Harris & Chandler, 1981), namely prevention of glomerular

damage without suppression of autoimmunity. In contrast to these thiol compounds, Cy A was immunosuppressive in NZB/W mice, as indicated by its inhibition of the response to SRC, measured 4 days after antigenic challenge.

In contrast to the suppression of the response to SRC, treatment with Cy A did not prevent the serological changes in female NZB/W mice associated with autoimmunity. There was no significant effect on the levels of circulating immune complexes and total IgM and IgG by treatment with Cy A, and this was associated with the continued deposition of immune complexes in the kidney. There was however some reduction in the levels of antibody to dsDNA, an important disease marker in NZB/W mice (Steward & Hay, 1976) which might indicate some, subtle alteration in the nature of the immune complexes in treated mice. A point of some interest was the significant increase in serum rheumatoid factors by the treatment with Cy A. This was not simply the result of prolonged life span since it occurred quite early in treatment. Although this increase in levels of rheumatoid factor could be secondary to the effect of Cy A on the immune system of NZB/W mice, they might play a protective role, e.g. by increasing antigen–antibody binding leading to larger complexes and more rapid clearance. It must be stressed however that this was somewhat remote in view of the lack of effect of Cy A on circulating immune complexes and their renal deposition.

The dose of Cy A used in the present studies was sufficient to suppress skin allograft rejection in the NZB/W mouse, tested before clinical onset of their immunoproliferative disease (personal observations), as has been found for other strains of mice (Borel & Meszaros, 1980). Results not reported showed that Cy A at an oral dose of 50 mg/kg body weight gave some protection to NZB/W mice but not to the same degree as 100 mg/kg which was still below the maximum dose tolerated without toxicity (150 mg/kg). In view of the lack of significant effects on humoral autoimmunity, effects on cell-mediated immune mechanisms could be responsible for the prevention of renal damage observed here. The proteinuria and glomerular proliferation associated with acute serum sickness appeared to be mediated by macrophages (Holdsworth, Neale & Wilson, 1981), and a similar mechanism could be responsible for the renal damage associated with the spontaneous immunoproliferative syndrome of NZB/W mice (Theofilopoulos & Dixon, 1979). Cy A is generally assumed to act preferentially on proliferating T cells (Borel *et al.*, 1977) decreasing antibody production to T cell-dependent antigens in rodents. It would therefore be reasonable to conclude that treatment with Cy A successfully prolonged the life span of female NZB/W mice by virtue of its selective effect on T lymphocytes, as indicated by suppression of the response to SRC. Thus T cells might play a role in activating monocytes and it has been indicated that Cy A '*in vitro*', can influence monocytes and prevent cytotoxic T cell responses perhaps by impairing release of interleukins-1 and -2, respectively (Bunjes *et al.*, 1981).

It has recently been shown (Neild *et al.*, 1983) that Cy A inhibited acute serum sickness nephritis, induced in rabbits by challenge with bovine serum albumin, without impairing the humoral response to this antigen, given with bacterial lipopolysaccharide as adjuvant. These results, in this induced model of glomerulonephritis, were thus very similar to the present results involving NZB/W female mice who spontaneously develop a similar renal condition. Based on the premise that Cy A was effectively protecting renal glomeruli by virtue of its preferential effect on T cells it would suggest that these cells have an important role in both induced and spontaneous renal disease, associated with immune complex deposition. As a corollary it is also implied that humoral autoimmunity in NZB/W mice may be thymic independent and this would be supported by the fact that such autoimmunity persists after impairment of T cells functions develop (Talal, 1976).

Dose related nephrotoxicity and hepatotoxicity occur in transplant patients given Cy A and this would counsel caution for its use in human SLE. In the present report, treatment with Cy A was begun before the onset of clinical disease and since this is not possible in man further studies in NZB/W mice with established autoimmunity are being done.

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