Clin. exp. Immunol. (1989) **78**, 449–453

Murine chronic graft-*versus*-host disease as a model of systemic lupus erythematosus: effect of immunosuppressive drugs on disease development

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(Accepted for publication 27 July 1989)

SUMMARY

The effect of a number of drugs commonly used to treat the more severe exacerbations of the autoimmune disease systemic lupus erythematosus (SLE) in humans has been investigated in the murine chronic graft-*versus*-host (GVH) induced model of lupus. This was undertaken in order to determine the value of this model for the investigation of immunomodulant drugs, with particular regard to the reproducibility of disease induction and methods of monitoring disease progression. The drugs were azathioprine, cyclophosphamide, cyclosporin A and dexamethasone. All of these, except for azathioprine, reduced disease severity, assessed as the development of lupus nephritis. Anti-ssDNA autoantibodies were also reduced in titre in the dexamethasone-treated group. Overall, these findings, combined with the reproducible induction of disease seen in this model, support the use of chronic GVH disease as a model for SLE and show that the induced disease can be ameliorated by drugs effective in the treatment of SLE in humans.

Keywords systemic lupus erythematosus immunosuppression

INTRODUCTION

Chronic graft-*versus*-host disease (GVHD) induced in C57BL/10 × DBA/2 F1 hybrid mice (B10.D2 F1) by inoculation of parental DBA/2 spleen cells, has been proposed as a model of the human autoimmune disease systemic lupus erythematosus (SLE) (Gleichmann, van Elven & van der Veen, 1982; Popovich & Bartlett, 1987; Bruijn *et al.*, 1988). The induced disease is characterized by the production of a range of autoantibodies similar to those seen in human SLE, including anti-ds and -ssDNA, anti-red blood cell, anti-T cell and anti-cytoskeletal autoantibodies (Rolink, Radaszkiewicz & Melchers, 1987). Autoantibodies reactive with extractable nuclear antigens (ENA) have also been described (Kimura *et al.*, 1987).

Pathological changes associated with this model include lymphoid infiltration of the salivary glands and liver and development of periarteritis (van Rappard-van der Veen *et al.*, 1983). The mice also develop severe glomerulonephritis, believed to be caused by the deposition of immune complexes in the kidney. The renal lesion is similar to human membranous lupus nephritis (Gleichmann *et al.*, 1982, Bruijn *et al.*, 1988).

This model offers significant advantages over the existing, spontaneous, murine models of SLE such as NZB/W F1 or MRL/Mp-lpr/lpr, as the disease can be reliably induced and follows a predictable, relatively short time course. These aspects of the GVHD model render it particularly suitable for testing drug effects (Popovich & Bartlett, 1987). In our hands, however, disease induction was significantly less than that reported in the literature.

Here we have investigated the reproducibility of disease induction in this model, and have shown that this can be significantly improved, without altering the underlying disease pathology, by pretreating recipient mice with 2′-deoxyguanosine. Methods of monitoring disease progression were also examined such that the effect of a number of immunosuppressive drugs, which have been used clinically to treat SLE, could be assessed in this model.

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MATERIALS AND METHODS

Animals

All mice were obtained from Harlan Olac at 8 to 12 weeks of age and were housed in cages of 10 with a 12 h light/dark cycle. Food (Labsure CRM diet) and water were given *ad libitum*.

Induction and monitoring of GVH-induced disease

Chronic GVHD was induced in C57BL/10 × DBA/2 F1 female mice by two i.p. inoculations of 10^8 DBA/2 spleen cells 1 week apart. In subsequent experiments recipient mice were pretreated with 50 mg/kg of 2′-deoxyguanosine (Sigma) given intraperitoneally 3 days prior to the first spleen cell inoculation.

The induced GVHD was monitored weekly, from 3 weeks post-inoculation, by measuring proteinuria as a marker of lupus nephritis, using Ames Uristix. These were read on the following 1–4 scale, where 1, 0·3 g/*l*; 2, 1 g/*l*; 3, 3 g/*l*; and 4, >20 g/*l* of

protein. The results are presented as mean values for each group of mice. To assess the incidence of lupus nephritis during the tests a proteinuria score >2 was considered indicative of disease.

A serum sample was collected from the tail vein of all mice at 7 weeks post-inoculation, and stored at −20°C before being assayed for anti-ssDNA autoantibodies by ELISA. Polyvinyl microtitre plates (Flow) were pretreated with 0·005% protamine sulphate (Sigma) dissolved in distilled water for 1 h at room temperature. After washing the plates in distilled water, heat-denatured DNA (Salmon testes, Sigma) at 1 μg/ml was added to the wells in citrate buffer, pH 8, and left overnight before washing in phosphate-buffered saline (PBS) containing 0·5% Tween 20 (PBS/Tween). Serum samples, diluted 1:200 in PBS/Tween, were then added to the wells and left for 2 h before washing. Bound antibody was then visualized using an alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin reagent (Sigma) diluted 1:1000 in PBS/Tween followed by *p*-nitrophenyl phosphate (Sigma) as substrate. Thirty minutes were allowed for colour development. A standard positive serum (MRL/Mp-1pr/1pr) and a negative normal mouse serum (B10.D2 F1) were included as controls on all plates. Results are presented as the optical density recorded at 405 nm.

Autoantibodies reactive with double stranded DNA were identified in 14 week serum by indirect immunofluorescence against *Crithidia lucilliae* as previously described (Gleichmann *et al.*, 1982).

Fourteen weeks after the induction of the GVHD, or before in the case of mice with severe lupus nephritis, all mice were killed, and the kidneys, liver and spleen collected in neutral buffered formalin for subsequent histological examination. A serum sample was also taken and albumen/globulin ratio, alpha-2 macroglobulin and total gamma-globulin were estimated by densitometry after zone electrophoresis. Normal, control values for the above parameters were obtained from a group of 47, age-matched, normal B10.D2 F1 hybrids.

Histological techniques

The organs collected at killing were fixed for 24 h in neutral buffered formalin before being blocked in paraffin wax. Sections (2 μm) were stained with haematoxylin and eosin and Jones silver methenamine. These were then examined microscopically and the severity of the renal lesion, assessed as basement membrane thickening and mesangial matrix expansion, was scored according to the following guidelines: 0, normal glomerulus; 1, mild glomerular basement membrane (GBM) thickening in 50% glomeruli; 2, more extensively thickened GBM in all glomeruli; 3, extensive thickening in all glomeruli, adhesions and sclerosis in <25% glomeruli; and 4, extensive sclerosis in >25% of glomeruli, end stage kidney.

An animal was considered diseased if the pathology score was ⩾1. Normal, control, B10.D2 F1 mice showed no pathological changes in the kidneys.

Experimental design

The efficacy of 2′-deoxyguanosine pretreatment was determined by comparing the disease parameters from 50 mice that had received 2×10^8 spleen cells alone with 61 mice that were given 2′-deoxyguanosine prior to the spleen cell inoculum.

Drug tests consisted of a disease control group of at least 10 mice in which the chronic GVHD had been induced but not treated. A second group of 10 mice formed the test group, treated with drug from 4 weeks post spleen cell inoculation until the end of the test at 14 weeks. Dexamethasone was obtained from Merck, Sharp and Dohme, cyclophosphamide from WB Pharmaceuticals, cyclosporin A (Sandimmune) from Sandoz and azathioprine from Sigma. The drugs were dissolved in saline plus 1·5% Tween 80 except for cyclosporin which was dissolved in olive oil, and administered daily at various doses. Azathioprine was administered intraperitoneally while other drugs were given *per os*.

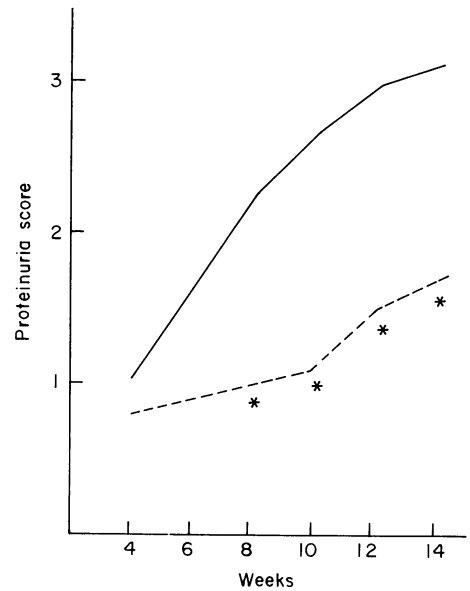

Fig. 1. Effect of 2′-deoxyguanosine pretreatment (——) on the appearance of proteinuria. (- - - -), No pretreatment. * Significantly different from pretreated group. $P < 0.05$, Mann–Whitney *U*-test.

Animals which developed severe nephrotic syndrome, identified by persistent proteinuria and ascites, were killed before the end of the test. Samples from these mice were included in the analysis of the test. In the case of proteinuria, the final score at killing was carried over into subsequent weeks to allow mean values to be calculated.

The selected disease parameters were compared between the disease control and the drug treated groups using the Mann–Whitney *U*-test for non-parametric values such as proteinuria scores and renal pathology and Student's *t*-test for serological data.

The incidence of disease was determined by establishing a cutoff point for a particular parameter, a value above which was considered diseased. For glomerular pathology a cutoff value of ⩾1 was established, while for proteinuria a score >2 was considered indicative of disease. Statistical significance was determined using Fisher's exact test.

RESULTS

The effect of 2′-deoxyguanosine pretreatment on disease incidence

The effect of using 2′-deoxyguanosine pretreatment, compared with spleen cells alone, on the appearance of proteinuria is shown in Fig. 1. Pretreatment with 2′-deoxyguanosine significantly increased the level of proteinuria from 8 weeks until the

Table 1. Effect of 2′-deoxyguanosine pretreatment on the development of graft-*versus*-host (GVH) induced lupus nephritis

Treatment	Number of mice	Albumen/globulin ratio	α_2-Globulins*	γ-Globulins*	Anti-ssDNA†	Pathology score	Incidence of renal disease‡
No pretreatment	50	1·09±0·16	19±6	7·9±1·8	0·2±0·1	1·0±0·4	42
50 mg/kg 2′-deoxyguanosine	61	0·45±0·14§	40±13§	6·3±2·0	0·4±0·2	2·1±0·3§	87§

Results are expressed as mean±s.d.
Chronic GVH disease was induced and monitored as described in the Materials and Methods section.
* % of total serum proteins.
† Optical density units at 405 nm.
‡ % of mice with renal pathology score ⩾1.
§ Significantly different from mice not receiving 2′-deoxyguanosine pretreatment ($P = < 0·05$).

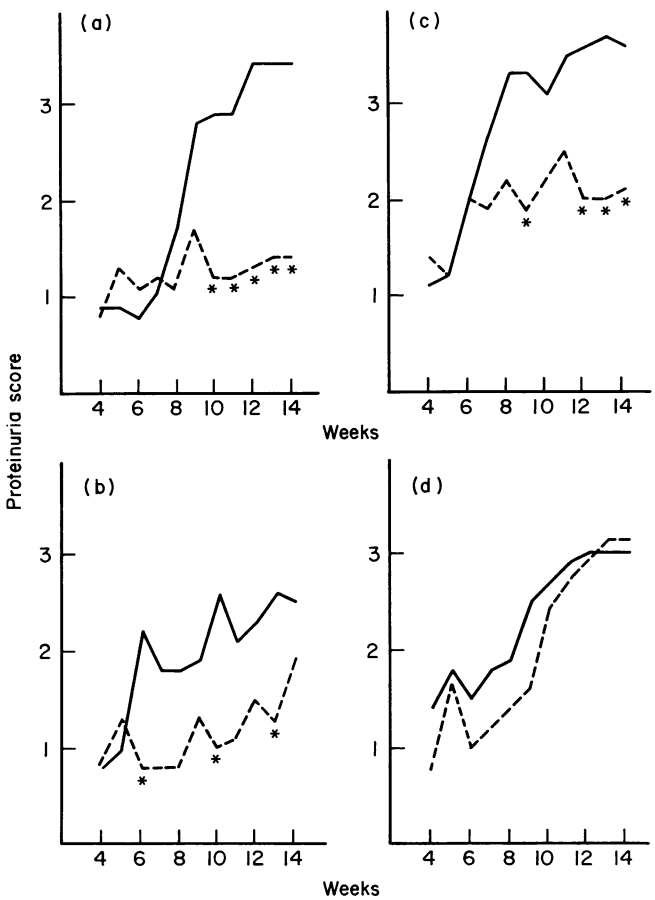

Fig. 2. Effect of drug treatment on the appearance of proteinuria in graft-*versus*-host (GVH) induced systemic lupus erythematosus. (——), Disease control; (- - - -) drug-treated. (a) Dexamethasone; (b) cyclophosphamide; (c) cyclosporin A; and (d) azathioprine. * Significantly different from disease control group. $P < 0·05$, Mann–Whitney *U*-test.

end of the test. A similar effect was seen on the serological parameters shown in Table 1; pretreatment significantly lowered the albumen/globulin ratio and increased the level of alpha-2 globulins. Gamma-globulin levels were unchanged while anti-ssDNA autoantibodies were raised, but not significantly. In a separate experiment antibodies reactive with *Crithidia* DNA were found in 90% of 2′-deoxyguanosine-treated mice.

Table 2. Incidence of proteinuria in mice with chronic graft-*versus*-host (GVH) induced systemic lupus erythematosus: effect of drug treatment

	Time (weeks) into test of proteinura sampling					
Treatment	4	6	8	10	12	14
Disease control*	6	6	60	80	87	93
Dexamethasone 1 mg/kg	11	22	11†	11†	22†	22†
Disease control	30	70	100	90	90	90
Cyclosporin A 70 mg/kg	40	43	66	66	55	55
Disease control	8	37	52	52	69	78
Cyclophosphamide 25 mg/kg	10	10	10†	20	30	50
Disease control	30	50	60	90	100	100
Azathioprine 5 mg/kg	55	37	50	75	87	87

Chronic GVH disease was induced and treated as described in Materials and Methods.
* Percentage of mice with proteinuria score >2.
† Significantly different from disease control; $P = < 0·05$, Fisher's exact test.

The incidence and severity of lupus nephritis were significantly increased following 2′-deoxyguanosine pretreatment. Microscopic examination of the diseased renal tissue revealed a similar, membranous lesion in both groups of mice, indicating that the 2′-deoxyguanosine treatment did not alter the underlying pathological changes associated with the GVHD.

On the strength of these findings, 2′-deoxyguanosine pretreatment was adopted in all further tests.

Drug effects on disease progression

Appearance of proteinuria. Drug effects on the appearance of proteinuria are shown in Fig. 2. In disease control (i.e. untreated) mice proteinuria began to increase above the normal background level (< 1 in normal B10.D2 F1 mice) around week 5 and by 12 weeks the majority of mice (>80%) had elevated proteinuria.

Dexamethasone was the most effective drug, as mean proteinuria values were only slightly above those of normal animals. Cyclosporin A did not completely suppress the appearance of proteinuria but did inhibit progression to the nephrotic levels seen in the disease control. The effect of cyclophosphamide was masked by the lower level of proteinuria

Table 3. Effect of drug treatment on serological and pathological parameters associated with chronic graft-*versus*-host (GVH) induced systemic lupus erythematosus

Treatment	Number of mice	Albumen/globulin ratio	α_2-Globulins*	γ-Globulins*	Anti-ssDNA†	Pathology score	Incidence of renal disease‡
Disease control	15	0·45±0·33	47±14	3·4±1·3	0·6±0·2	2·1±0·7	93
Dexamethasone 1 mg/kg	9	1·40±0·40§	12±8§	3·5±0·7	0·3±0·3§	0·5±0·6§	11§
Disease control	10	0·42±0·32	21±16	5·7±2·1	0·6±0·3	2·3±0·4	100
Cyclosporin 70 mg/kg	9	0·93±0·48	17±14	7·9±4·3	0·5±0·2	1·5±1·3	67
Disease control	20	0·59±0·49	37±22	7·2±2·0	0·3±0·2	1·7±1·2	70
Cyclophosphamide 25 mg/kg	10	1·40±0·56§	16±20	6·1±0·8	0·2±0·1	0·9±0·9	50
Disease control	7	0·24±0·10	55±7	6·5±3·1	0·3±0·1	2·4±0·5	100
Azathioprine 5 mg/kg	7	0·14±0·10	60±7	7·0±2·1	0·4±0·2	2·1±0·6	100
Normal controls	47	1·70±0·30	7±1	6·6±0·80	<0·10	<0·5	0

Results are expressed as mean±s.d.
* % of total serum proteins.
† Optical density units at 405 nm.
‡ % of mice with renal pathology score ⩾1.
§ Significantly different from disease control ($P<0·05$).

developed in this test but the drug significantly reduced proteinuria especially during the early part of the experiment. Azathioprine had no effect on proteinuria at the dose given.

When the incidence of raised proteinuria (percentage of mice with a proteinuria score >2) was examined (Table 2) the reproducibility of the GVHD-induced model of lupus was clearly demonstrated. In all four tests reported here, >75% of the untreated control mice had developed lupus nephritis, evidenced by raised proteinuria, by 14 weeks. However, the disease process could still be modified by drug treatment, as dexamethasone significantly reduced the incidence of proteinuria. Cyclosporin A also lowered the incidence of proteinuria as did cyclophospamide but neither achieved statistical significance. Azathioprine was inactive.

Serological changes. GVHD-induced SLE causes major changes in serum protein profiles due to the development of glomerulonephritis and nephrotic syndrome. These changes can be seen in the serum profiles for the disease control groups shown in Table 3. They are characterized by a low albumen/globulin ratio, caused by loss of serum albumen (data not shown) and raised alpha-2 macroglobulin levels.

Treatment with dexamethasone significantly improved the serology while cyclosporin A and cyclophoshamide both reduced the severity of the changes but neither achieved significance. Azathioprine treated animals were similar to the disease control, with no improvement in serology.

Chronic GVHD is also characterized by the appearance of serum autoantibodies. The levels of anti-ssDNA autoantibodies, measured at 7 weeks, are shown in Table 3. Only dexamethasone significantly lowered the levels of serum autoantibodies. Anti-ssDNA activity was not detected in normal control animals.

The serum level of total gamma-globulin at the end of each test was also estimated. In the dexamethasone test both treated and disease control mice had low immunoglobulin levels while there was no difference between treated, diseased and normal mice in the other tests.

Renal pathology

The incidence and severity of the lupus nephritis in diseased and treated animals are shown in Table 3. In untreated mice the majority (on average >90%) exhibited pathological changes as would be expected from the proteinuria data. Following treatment with dexamethasone both the incidence and severity of the renal lesions were significantly reduced. Cyclosporin A and cyclophosphamide also reduced renal disease, but not significantly. Azathioprine had no effect on the renal disease, as expected from the other data.

DISCUSSION

The aims of this study were firstly to investigate the reproducibility of the chronic GVHD-induced model of SLE; then to develop methods of monitoring disease progression in the model; and finally to use these to ascertain whether the induced disease could be successfully treated by drugs employed to treat SLE in humans.

The major advantages of the GVHD model are its reproducibility, reliability and short time course, Compared with the spontaneous NZB/W model, in which only 50% of mice developed renal disease within 10 months (Theophilopoulos & Dixon, 1985), the GVHD model showed a 70% incidence of lupus nephritis in 4 months (van Rappard-van der Veen *et al.*, 1983). In our hands, however, this level of disease was not routinely obtained. This may reflect differences between the DBA/2 cell inocula given to induce disease; van Rappard-van der Veen *et al.* (1983) and Bruijn *et al.* (1988) used a mixture of spleen and lymph node cells, whereas we used spleen cells alone. Disease induction following spleen cell inoculation was improved by pretreating the recipient mice with 2′-deoxyguanosine. The mechanism by which this treatment enhanced disease induction was not investigated here, although the drug is known to reduce T suppressor cell activity (Dosch *et al.*, 1980) and such cells have been shown to impair the GVHD reaction in

untreated B10.D2 F1 mice (Rolink, Gleichmann & Gleichmann, 1983). It seems logical therefore to assume that 2'-deoxyguanosine has lowered host T suppressor cell function and allowed a more vigorous GVHD reaction to develop. This treatment did not appear to affect the underlying disease process as the pathological changes seen in the kidney were the same in both treated and untreated mice. Likewise, similar autoantibodies, including the anti-native dsDNA characteristic of SLE, were seen in treated mice.

Lupus nephritis is a major cause of morbidity in patients with SLE (Pollak, 1976) and for this reason it was chosen as the yardstick by which drug effects were measured in this model. The membranous nephropathy of GVHD-induced SLE is always associated with the development of nephrotic syndrome (Gleichmann *et al.*, 1982; Bruijn *et al.*, 1988) and this can be easily monitored by measuring changes in serum proteins. The renal lesion in the GVHD model is extremely reproducible, with >90% of mice showing measurable pathological changes in the kidneys by 14 weeks and this allows the development of the nephrotic syndrome to be used as a reliable marker of disease progression.

The results presented in this paper clearly showed that the induced SLE-like disease could be successfully treated with immunosuppressive drugs. To avoid drug treatment suppressing the initiating GVH reaction, we began treatment at 28 days, by which time the majority of GVH mice had autoantibodies in their serum (Gleichmann *et al.*, 1982) and the disease process was well established. This has the additional advantage of being closer to the clinical situation in that the disease is already established before treatment is begun. It will also identify drugs that are capable of affecting an ongoing disease process. This delay in treatment probably accounts for the reduced activity shown by cyclophosphamide in our model compared with the results obtained by Popovich & Barlett (1987).

The drugs that we chose to test in this model have been used to treat SLE in both humans and in the murine NZB/W F1 model (Theophilopoulos & Dixon, 1985; Burlingame & Delafuente, 1988). In common with these, the GVHD model responded to both steroid and cyclophosphamide treatment while cyclosporin A was less effective. Azathioprine was unexpectedly inactive but this could be explained by the proliferative nature of the renal lesion seen in the NZB/W F1 and in many of the human lupus nephritides, where a direct anti-proliferative effect of azathioprine may help to preserve renal integrity. In the GVHD model the renal lesion is of a membraneous type, with only a minor proliferative component confined to the mesangial matrix (Gleichmann *et al.*, 1982), therefore an anti-proliferative drug would have little effect, as indeed was the case.

The mode of action of the drugs was not investigated in this study although it is probable that they exert their effect by the suppression of autoantibody synthesis, as has been postulated in previous studies using the NZB/W F1 model (Gunn, 1986). Serological data did not appear to support this hypothesis as two of the three treated groups with a reduction in renal disease showed no consistent suppression of total gamma-globulin or autoantibodies to ss-DNA. The single timepoint measurements employed to monitor such changes at 7 and 14 weeks of the test may not, however, give a complete picture of humoral immunosuppression following drug treatment.

Chronic GVHD provides a reliable and reproducible model of SLE which can be used to test drug effects on disease progression. No animal model can cover all facets of such a protean disease as SLE and such a model, if it existed, would be by definition poorly reproducible and difficult to assess and hence of little value for testing therapeutic drugs. The GVHD model offers an acceptable compromise between reproducibility and response to treatment and the close analogy with a human disease.

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