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Long term administration of cyclophosphamide into MRL/1 mice. II. The effects on the isotype of anti-DNA antibodies and immunoglobulin secreting cells in the spleen

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SUMMARY

Weekly injections of cyclophosphamide (Cy) at a dose of 20 mg/kg body weight prevented IgM to IgG class switch of serum anti-DNA antibodies and also immunoglobulin secreting cells in the spleen of MRL/Mp-*lpr/lpr* (MRL/1) mice. Culture experiments revealed that splenic B cells of Cy treated mice gave rise to more IgM and less IgG secreting cells than those of untreated mice in response to lipopolysaccharide. These results suggested that Cy suppressed enhanced differentiation of B cells into IgG secreting cells in MRL/1 mice, which would result in reduction of IgG anti-single stranded DNA antibodies and improvement of murine lupus like syndrome.

Keywords cyclophosphamide MRL/l mice anti-DNA antibodies

INTRODUCTION

MRL/1 mice with lupus like syndrome show various immunological abnormalities (Murphy & Roths, 1978; Theofilopoulos *et al.*, 1980a). The extraordinary number of immunoglobulin secreting cells (IgSC) in the spleen, particularly IgGSC, is one of the prominent features of older MRL/1 mice (Theofilopoulos *et al.*, 1980b). Recently, IgM to IgG class switch of anti-DNA antibodies was shown to correlate with disease activity in NZB/NZW F_1 mice (Steward & Hay, 1976; Papoian, Pillarisetty & Talal, 1977) or in patients with systemic lupus erythematosus (SLE) (Pennebaker, Gilliam & Ziff, 1977). In this study, we examined the effect of Cy on isotype conversion of anti-DNA antibodies and IgSC in the spleen of MRL/1 mice. We also studied the isotype of IgSC generated in response to lipopolysaccharide (LPS) *in vitro*.

MATERIALS AND METHODS

Mice. Male MRL/1 and MRL/Mp−+/+ (MRL/n) mice were used, obtained from The Jackson Laboratory, Bar Harbor, Maine, USA and maintained at our animal facilities.

Treatment with Cy. Cy (Endoxan; Shionogi Seiyaku Co., Osaka) was injected i.p. at a dose of 20 mg/kg once a week from 5 weeks of age until 1 week before the sacrifice.

Anti-DNA antibodies. Serum levels of anti-single stranded DNA (ssDNA) antibodies were

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measured by enzyme linked immunosorbent assay as described in the preceding report (Shiraki, Fujiwara & Tomura, 1984). IgG and IgM anti-ssDNA antibodies were measured by applying heavy (μ or γ) chain specific antisera conjugated with peroxidase (Cappel Laboratories, Cochranville, Pennsylvania, USA). Titre of each sample was determined by referring to standard serum, which was determined to contain 100 u/ml IgG and IgM anti-ssDNA antibodies. Standard serum consisted of selected sera from older MRL/1 mice with high levels of anti-ssDNA antibodies. Statistical analyses were performed by Student's *t*-test.

Enumeration of IgSC. The number of IgGSC and IgMSC was estimated by reverse haemolytic plaque assay (Gronowicz, Coutinho & Melchers, 1976), using heavy chain specific antisera (Medical and Biological Laboratories, Nagoya). For the detection of IgSC in the preliminary experiment, polyvalent anti-mouse immunoglobulin antisera were used for developing plaques.

Preparation of B and T cells. Spleen cell suspension was prepared by mincing spleen on a stainless mesh (No. 200), using minimum essential medium (MEM; Nissui Seiyaku Co., Tokyo) containing 5% fetal calf serum (FCS; Reheis Chemicals, Phoenix, Arizona, USA) and 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES; Sigma Chemicals, St Louis, Missouri, USA). Spleen cells from two or three mice of each group were pooled and B or T cell enriched populations were obtained as follows. B cells were prepared by treating spleen cells with rabbit anti-Thy-1 antibodies and guinea-pig complement, followed by the passage through a Sephadex G-10 column to remove dead cells as well as antibody producing cells already present in the cell suspension (Fujiwara & Akiyama, 1980). T cells were prepared by passage of spleen cell suspension over a nylon wool column, following the procedures described by others (Julius, Simpson & Herzenberg, 1973).

Cell culture experiments. For the culture of spleen cells, RPMI 1640 (GIBCO, Grand Island, New York, USA) adjusted to pH 7·2 with 7% $NaHCO_3$ and supplemented with 2 mM L-glutamine and 10% FCS was used. B and T cells obtained from each group of mice were adjusted to 5×10^6 viable cells per ml and 0·1 ml of each was cultured in various T–B cell combinations in a microplate (Microplate; Falcon Plastics, Oxnard, California, USA) under humidified air containing 5·5% CO_2 in the presence of 1 μg/well LPS (*E. coli* 0111; B4, DIFCO Laboratories, Detroit, Michigan, USA). After the culture for 3 days, IgSC generated in each well were enumerated.

RESULTS

Isotype of serum anti-DNA antibodies

The development of IgG and IgM anti-ssDNA antibodies with age in Cy treated and untreated MRL/1 mice are shown in Fig. 1. Cy suppressed the development of IgG antibodies more prominently than IgM antibodies. Cy treatment decreased IgG antibodies to almost 7% of those in

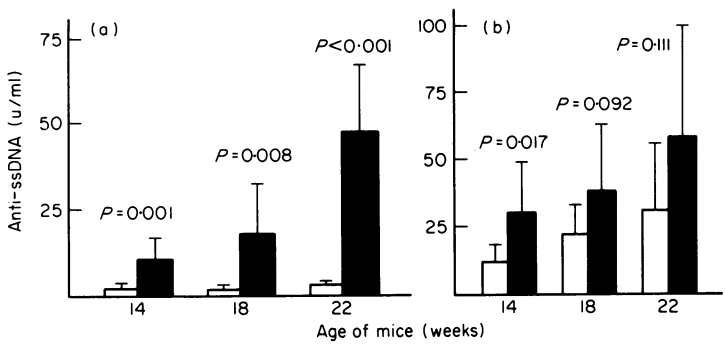

Fig. 1. The effect of Cy on the development of (a) IgG or (b) IgM anti-ssDNA antibodies. Each column represents mean ± s.d. of 9–13 mice. *P* value refers to the statistical significance between Cy treated (□) and untreated (■) mice.

untreated mice at 22 weeks of age, while IgM antibodies of Cy treated mice were more than 50% of levels of untreated mice.

Isotype of IgSC in the spleen

The number and isotype of IgSC in the spleen was determined at 14 and 22 weeks of age (Table 1). The congenic strain MRL/n mice, which lack *lpr* gene and do not manifest lymphoproliferation (Murphy & Roths, 1978), were included for comparison. Progressive increase of IgSC, particularly IgGSC, was noted in untreated MRL/1 mice and the ratio of IgGSC/IgMSC also increased with age. Administration of Cy effectively suppressed the development of IgGSC in the spleen of MRL/1 mice so that the ratio of IgGSC/IgMSC did not increase with age in Cy treated mice. The number of IgGSC as well as IgMSC of Cy treated MRL/1 mice at 22 weeks of age was not significantly different from that of MRL/n mice.

Table 1. The isotype of IgSC in the spleen of Cy treated and untreated MRL/1, and MRL/n mice

		Age of mice (weeks)	
		14	22
Group of mice		IgSC/spleen ($\times 10^4$)	(mean ± s.d.)
Cy treated MRL/1	IgGSC	34·5 ± 10·7	26·5 ± 8·2
	IgMSC	30·5 ± 8·2	31·6 ± 12·1
	ratio	1·2 ± 0·4	0·9 ± 0·3
Untreated MRL/1	IgGSC	216·0 ± 94·5	1,123·0 ± 566·5
	IgMSC	48·4 ± 20·9	126·2 ± 82·0
	ratio	5·1 ± 3·2	11·5 ± 6·6
MRL/n	IgGSC	5·1 ± 4·5	26·7 ± 21·6
	IgMSC	7·3 ± 2·4	17·3 ± 11·3
	ratio	0·6 ± 0·4	1·4 ± 0·4

IgSC in the spleen enumerated by reverse haemolytic plaque assay using protein A coated target cells and the ratio of IgGSC/IgMSC of each mouse was estimated. Each value represents mean ± s.d. of 5–13 mice.

In vitro *generation of IgGSC in response to LPS*

As a preliminary experiment, helper effect of T cells in the generation of IgSC in response to LPS was examined. Spleen cells of 24 week old MRL/1 mice were separated into B and T cells and constant number of B cells (5×10^5 cells/well) were cultured for 3 days with various number of T cells. The number of IgSC generated in each well was estimated, using polyvalent anti-mouse immunoglobulin antisera for developing plaques. As shown in Fig. 2, the number of IgSC generated in response to LPS remarkably increased in the presence of T cells. Then, 10, 24 and 32 week old mice, either Cy treated or untreated, were used. The number of IgGSC and IgMSC was estimated using class specific antisera, respectively. As shown in Fig. 3, the number of IgSC generated in response to LPS remarkably decreased with age in both Cy treated and untreated mice. The number of IgMSC was almost 10 times as much as IgGSC in 10 week old mice. The ratio of IgGSC/IgMSC increased with age in untreated mice, while it remained in low value in Cy treated mice. It was especially noted that B cells of Cy treated mice (B[Cy]) gave rise to more IgMSC and less IgGSC than B cells of untreated mice (B[UT]) in the presence of T cells of any sources. This feature was not apparent in 10 week old mice and came to be evidently admitted in 24 and 32 week old mice, though the number of total IgSC became far decreased at 32 weeks of age.

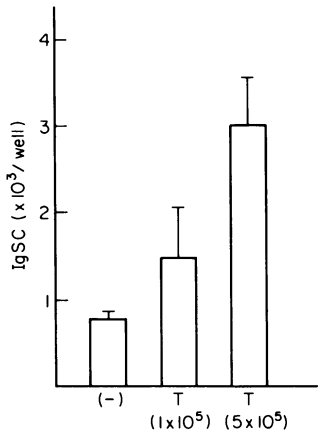

Fig. 2. Helper effect of various number of T cells in the generation of IgSC in response to LPS in 24 week old MRL/1 mice. Various number of T cells were added to a constant number of B cells (5×10^5 cells/well). Each column represents the mean ± s.d. of triplicate experiments.

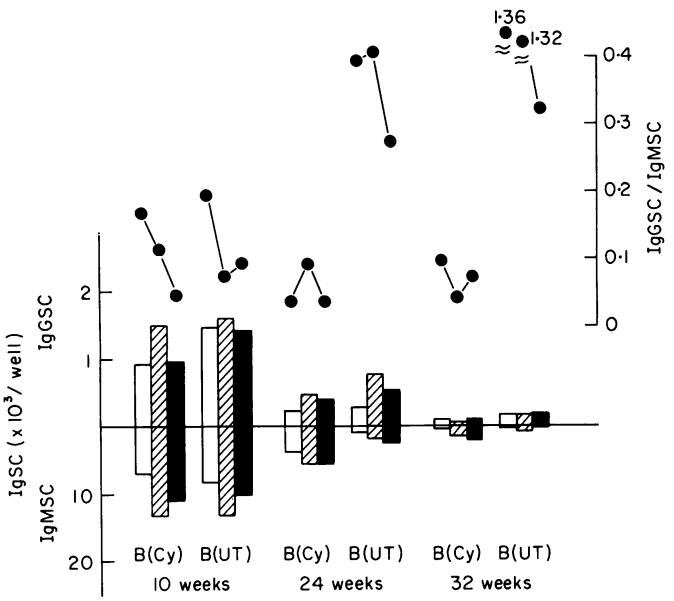

Fig. 3. The number of IgG and IgMSC generated in the culture of T and B cells in response to LPS. Each column represents mean value of triplicate experiments. The mean value of the ratio of IgGSC/IgMSC is plotted over each column. B or T (Cy): B or T cells obtained from Cy treated mice. B or T (UT): B or T cells obtained from untreated mice. □ = T(−); ▨ = T(Cy); ■ = T(UT).

As for T cells, those of both Cy treated and untreated group of mice enhanced the generation of IgSC in response to LPS. No evidence of the effect of Cy on helper activity of T cells could be admitted in these short term culture experiments.

DISCUSSION

Cy effectively prevents the development of murine lupus like syndrome in MRL/1 mice and markedly prolongs their survival (Shiraki *et al.*, 1984). In this study, we concentrated on isotype

conversion of anti-DNA antibodies and IgSC in the spleen of MRL/1 mice. MRL/1 mice are characterized by the early onset of murine lupus like syndrome with high levels of anti-DNA antibodies. Recently, sequential switch of isotype of anti-DNA antibodies from IgM to IgG type was shown to correlate with the disease activity in NZB/NZW F_1 mice (Steward & Hay, 1976; Papoian *et al.*, 1977). In human cases, Koffler *et al.* (1969) found a predominance of IgG deposits in the kidneys of patients with more severe lupus nephritis. Pennebaker *et al.* (1977) also investigated 36 SLE patients and demonstrated that patients with IgG type anti-native DNA were suffered from more active disease than those with IgM antibodies. As a whole, these observations suggest that IgG anti-DNA antibodies are more important in, or at least highly related with, the pathogenesis of lupus nephritis. In our study, Cy was shown to decrease IgG anti-ssDNA antibodies more prominently than IgM antibodies. It is suggested that improvement of lupus nephritis in Cy treated mice should be ascribed to decrease in IgG anti-ssDNA antibodies.

MRL/1 mice develop a large number of IgSC in the spleen with increase of age. The isotype of IgSC also converts from IgM to IgG type (Theofilopoulos *et al.*, 1980b; unpublished observations). Again, Cy preferentially diminished IgGSC, as evidenced by decreased ratio of IgGSC/IgMSC in Cy treated mice. These results are consistent with the report that 7S antibody response in rodents were more susceptible to suppression by Cy than 19S antibody response in certain experimental conditions (Santos & Owens, 1966). Culture experiments revealed that B cells from Cy treated mice gave rise to more IgMSC and less IgGSC than those of untreated mice in response to LPS. These results raised the possibility that Cy prevented enhanced differentiation of B cells into IgGSC, which would result in the relative increase of B cell population to generate IgMSC in response to LPS.

T cells enhanced polyclonal activation of B cells by LPS, as also reported previously by others (Goodman & Weigle, 1979; Theofilopoulos *et al.*, 1980b). T cells of either group of mice increased IgSC generated in response to LPS *in vitro*. This enhancing effect of T cells is not due to contamination of a small number of B cells in T cell preparation, because the culture of nylon wool passed cells alone gave a very small number of IgSC, i.e. less than 7% of that with B cells. MRL/1 mice are characterized by marked proliferation of T cells, which exert excessive helper activity to induce IgSC in response to LPS *in vitro* (Theofilopoulos *et al.*, 1980b). In our study, administration of Cy did not alter the helper activity of T cells to induce IgMSC or IgGSC. Still, the effect of Cy on T cells *in vivo* should not be disregarded because Cy effectively suppressed the excessive proliferation of Thy-1 positive cells (Shiraki *et al.*, 1984). It might be possible that Cy reduced helper activity of T cells *in vivo* by reducing total number of T cells.

Cy seems to have dual actions on hyperplasia of lymphoid cells in MRL/1 mice. One is cytotoxic action on lymphoid cells, especially on excessively proliferating T cells, which might affect the amount and isotype of immunoglobulins spontaneously produced *in vivo*. The other is direct action on B cells. Cy was shown to suppress the accelerated differentiation of B cells into IgGSC in MRL/1 mice, which would result in reduction of IgG anti-ssDNA antibodies and improvement of lupus nephritis.

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