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Qualitative difference of anti-DNA antibody-producing cell precursors in the pre-immune B cell repertoire between normal and lupus-prone mice

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SUMMARY

The precursor frequency for anti-DNA antibody-producing cells in the pre-immune B cell repertoire was investigated in young female BALB/c and NZW mice, and in young and aged female NZB × NZWF1 (B/WF1) mice. Spleen cells from these mice were diluted serially and stimulated polyclonally *in vitro* with lipopolysaccharide (LPS) and IL-4 to induce both IgM and IgG1 production. The results demonstrated that there existed virtually no difference in precursor frequency for IgM anti-DNA antibody-producing cells between normal and lupus mice, confirming previous observations made by other investigators. In contrast, the number of precursors for IgG1 anti-DNA antibody-producing cells was much higher in young and old B/WF1 mice than in normal mice. These results suggest that the high frequency of precursors for IgG1 anti-DNA antibody-producing cells in the pre-immune B cell repertoire of B/WF1 mice is a crucial factor for the pathogenesis of systemic lupus erythematosus.

Keywords anti-DNA antibodies pre-immune repertoire NZB/WF1 mice IL-4

INTRODUCTION

Anti-DNA antibody has been considered to be one of the characteristic autoantibodies associated with pathological manifestations of human and murine systemic lupus erythematosus (SLE). However, it has been shown that anti-DNA antibodies can be induced even in normal mice when spleen cells of these mice are stimulated with B cell mitogens (Izui *et al.*, 1977; Sawada *et al.*, 1977). Therefore, the pathogenesis of this autoantibody could not simply be ascribed to the reactivity with DNA. The importance of the specificity, affinity, isotype or isoelectric points of the antibody have all been pointed out (Steward & Hay, 1976; Winfield, Faiferman & Koffler, 1977; Kohno *et al.*, 1983; Yoshida *et al.*, 1985). Especially, anti-DNA antibodies of IgG class are shown to be closely associated with the occurrence of lupus nephritis (Papoian, Pillarisetty & Talal, 1977; Slack *et al.*, 1984). It is important, therefore, to clarify the mechanism of IgG anti-DNA antibody production in lupus-prone animals.

Several groups of researchers have tried to determine the precursor frequency for anti-DNA antibody-producing cells in the B cell repertoire of lupus-prone mice (Pisetsky & Caster, 1982; Conger, Pike & Nossal, 1987). However, no significant difference was found in the frequency of IgM anti-DNA antibody-producing cell precursors in the splenic B cell repertoire between normal and lupus mice. In our previous work (Tsubata *et al.*, 1988), we compared the precursor frequency for high-affinity IgM anti-DNA antibody-producing cells between normal and lupus mice, and found that such precursors existed in the pre-immune repertoire of lupus mice at much higher frequency than in that of normal mice, whereas in the pre-receptor repertoire, which is constructed by the collection of germline gene segments encoding immunoglobulin molecules (Holmberg *et al.*, 1986), the frequency was similar among normal and lupus mice.

The aim of this study was to compare the frequency of precursors for high affinity anti-DNA antibody, which would react with DNA after isotype switch to IgG, producing cells in the pre-immune B cell repertoire between normal and lupus mice. IL-4 is known to enhance strikingly the production of IgG1 from uncommitted spleen B cells stimulated with bacterial lipopolysaccharide (LPS) by promoting these B cells to switch to IgG1 producing cells *in vitro* (Isakson *et al.*, 1982; Noma *et al.*, 1986; Bergstedt-Lingqvist *et al.*, 1988). Using a limiting dilution assay carried out in the presence of LPS and IL-4, McHeyzer-Williams & Nossal (1988) compared the reactivity with several antigens of IgM and IgG1 produced by B cells derived from single B cells in the pre-immune repertoire of normal mice. Their results indicated that the frequency of precursors for IgM reactive with intracellular antigens was high, whereas that of precursors for IgG1 reactive with the same intracellular antigens was very low. A similar limiting-dilution assay was used in the

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present study to investigate the precursor frequency for anti-DNA antibodies of IgM and IgG1 classes in the preimmune B cell repertoire of normal and NZB × NZWF1 (B/WF1) mice. The results show that the precursor frequency for IgM anti-DNA antibody in the pre-immune B cell repertoire is virtually the same among normal and lupus mice, whereas that for IgG1 anti-DNA antibody-producing cells is much higher in the pre-immune repertoire of young and old B/WF1 mice than in that of normal mice.

MATERIALS AND METHODS

Animals

BALB/c, NZW, and B/WF1 mice were obtained from facilities in our laboratories. C57BL10/ScCr (B10Cr) mice, which are LPS-non-responder mice (Coutinho & Meo, 1978), were originally obtained from Bomhoilgaad (Ry, Denmark) and maintained in our animal facility. B10Cr mice were used at 4–6 weeks of age as filler cell donors.

Culture conditions

RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 15% fetal calf serum (FCS) (M. A. Bioproducts, Walkersville, MD), 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (0·1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) was used. All cultures were performed at 37°C in a humidified atmosphere of 7·5% CO_2 in air.

Cell preparation

Spleen cell suspensions were prepared by teasing spleens, passing them through a stainless steel mesh sieve and sterilized cotton wool to remove cell clumps, and then removing erythrocytes by ammonium chloride treatment.

Limiting dilution assay

The culture system developed by Anderson, Coutinho & Melchers (1977) was used for limiting dilution analysis, with slight modifications. Briefly, graded numbers of spleen cells were cultured together with 60 μg/ml LPS (*Escherichia coli* 055:B5; Difco, Detroit, MI) in 0·2 ml of the medium in 96-well flat-bottomed plates (Costar, Cambridge, MA) in the presence or absence of 1% (v/v) of the culture supernatant from X63-Ag8.653 cells transfected with murine IL-4 cDNA (X63-Ag8.653 mIL-4 2-39, kindly provided by Dr H. Karasuyama, Tokyo University) (Karasuyama & Melchers, 1988). As filler cells, thymocytes from B10Cr mice passed twice through a nylon wool column were used at 6×10^5 cells/well. Thymocytes treated as described above gave only neglible background immunoglobulin. After 7 days of culture, aliquots of supernatants were removed and IgM, IgG, and IgG1, and anti-DNA antibodies of these classes were measured.

Radioimmunoassay

The measurement of anti-DNA antibodies of IgM, IgG, and IgG1 classes was carried out by solid-phase radioimmunoassay (RIA) as described by Eaton, Schneider & Schur (1983), with slight modifications. Flat-bottomed 96-well microtitre plates (Falcon 3912; Becton Dickinson, Oxnard, CA) were coated with 50 μl of 10 μg/ml heat-denatured calf thymus DNA (Worthington Biochemical Corp., Freehold, NJ) in phosphate-buffered saline (PBS). After blocking with 1% bovine serum albumin (BSA) in PBS, 50 μl of samples diluted with 1% BSA in PBS were applied for an overnight incubation at 4°C. After washing, wells were incubated with ^{125}I-labelled (Amersham International, Amersham, UK) goat anti-mouse μ chain (prepared and purified in our laboratory), goat anti-mouse γ chain (Zymed Lab., South San Francisco, CA), and goat anti-mouse γ1-chain-specific antibodies (Sera Lab., Crawley Down, UK) for the measurement of IgM, IgG, and IgG1 anti-DNA antibodies, respectively. After a 2-h incubation at room temperature, wells were washed and bound radioactivity was counted with a gamma counter.

The amounts of IgM, IgG, and IgG1 in culture supernatants were measured by sandwich RIAs using goat anti-mouse μ chain, γ chain, and γ1 chain-specific antibodies, respectively. These antisera were shown to be highly specific (data not shown). Radioactivities of wells above the mean counts of the background cultures + 3 s.d. were considered positive.

Statistical analysis

Poissonian analysis (Lefkovits & Waldmann, 1979) was used to determine the frequency of antibody-producing cell precursors.

RESULTS

Induction of B cell precursors for IgG1 producers by IL-4

To investigate the activity of IL-4 to induce IgG1 production in B cells of lupus mice, spleen cells from unimmunized 2-month-old BALB/c mice, and 2-month-old and 10-month-old B/WF1 mice were serially diluted, and cultured with LPS in the presence or absence of IL-4. IgM and IgG1 in the culture supernatants was assayed on day 7, and the wells containing significant levels of IgM and/or IgG1 were determined. The proportion of negative wells are plotted in Fig. 1. In all groups of mice examined, the precursor frequency for IgM-producing cells was not affected by the addition of IL-4. In contrast, the frequencies for IgG1-producing cells increased up to about 10 times in all three groups by the addition of IL-4. The proportions of IgM-producing cells in splenocytes, when cultured with LPS and IL-4, were 1/15, 1/7, and 1/9, and those of IgG1 producing cells were 1/27, 1/24, and 1/26, in 2-month-old BALB/c, 2-month-old B/WF1, and 10-month-old B/WF1 mice respectively.

Since the percentages of B cells in spleen cells of these mice were 36%, 30%, and 55%, respectively, the results indicated that more than 20% of spleen B cells from all tested mice produced IgM and one out of two to one out of four of them switched to IgG1, and that the frequency of isotype switch to IgG1 is not different between normal and lupus-prone mice in this assay system. Since in our preliminary experiments it was shown that IgM was also detectable in the majority of IgG1 positive wells in the culture of 2-month-old BALB/c, 2-month-old B/WF1, and 10-month-old B/WF1 splenic B cells at dilutions giving less than one antibody-producing cell precursors per well (data not shown), the isotype switch to IgG1 is suggested to occur *in vitro*.

Frequencies of precursor cells for IgM and IgG1 anti-DNA antibodies in the pre-immune B cell repertoire

The spleen cells from unimmunized 2-month-old female BALB/c (Igh^a) and NZW (Igh^c) mice, and 2-month-old as well as 10-month-old female B/WF1 (Igh^c) mice were cultured at limiting dilution in the presence of LPS and IL-4. Precursor frequencies for IgM- and/or IgG1-producing cells, and those for IgM anti-

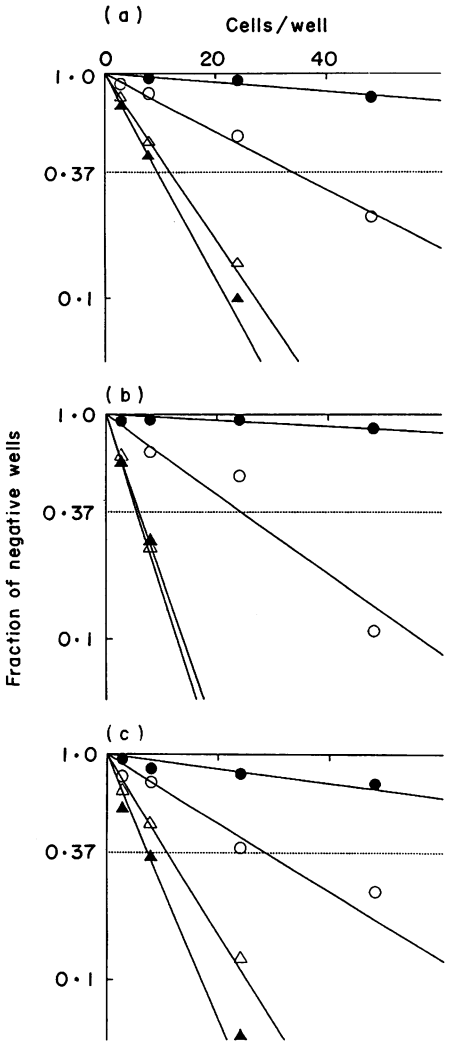

Fig. 1. IL-4 increased IgG1-secreting cells induced by LPS in normal and lupus mice. Spleen cells from 2-month-old BALB/c (a), 2-month-old B/WF1 (b), or 10-month-old B/WF1 (c) mice were cultured at limiting dilution together with 6×10^5 of nylon wool-passed thymocytes of B10Cr mice in the presence of LPS (60 μg/ml). (IgM, ▲; IgG1, ● or LPS plus 1%(v/v) of IL-4 containing culture supernatant (IgM, △; IgG1, ○). Immunoglobulin synthesis was measured after 7 days of cultures by solid-phase RIA.

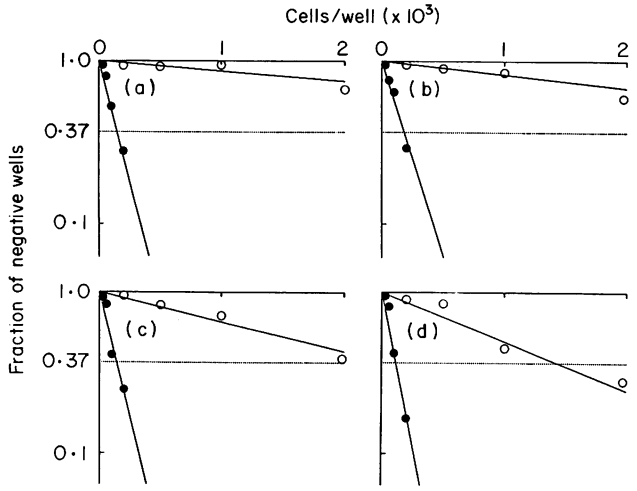

Fig. 2. Limiting dilution analysis of IgM and IgG1 anti-DNA antibody of 2-month-old BALB/c (a), 2-month-old NZW (b), 2-month-old B/WF1 (c), and 10-month-old B/WF1 mice (d). Spleen cells from each group of mice were cultured at limiting dilution for 7 days together with 6×10^5 of nylon wool-passed thymocytes of B10Cr mice in the presence of LPS (60 μg) plus 1% (v/v) of IL-4-containing culture supernatant. Production of IgM (●) and IgG1 (○) anti-DNA antibodies was determined by solid-phase RIA.

DNA antibody- and/or IgG1 anti-DNA antibody-producing cells were determined by using Poissonian analysis, and the ratios of the precursor frequency for anti-DNA antibody-producing cells to that for immunoglobulin-producing cells were calculated to estimate the frequency of anti-DNA antibody-producing cell precursors. Three mice were analysed per group of mice and the representative results are shown in Fig. 2 and Table 1. Of the IgM producing clones from all groups of mice, 7–8% secreted IgM anti-DNA antibodies, suggesting that there exists virtually no difference in the precursor frequencies for IgM anti-DNA antibody producers between normal and lupus-prone mice. This observation confirms previous findings by Pisetky & Caster (1982) and Conger *et al.* (1987), in spite of the fact that the proportion of IgM anti-DNA antibody-producing cell precursors to all IgM-secreting cells is higher than those in previous findings. The difference may be due to the fact that many of these anti-DNA antibodies seems to be low-affinity ones and that RIA for IgM anti-DNA antibody is more sensitive than that for IgM in our study. In contrast, the ratios of IgG1 anti-DNA antibody-producing cells to IgG1-producing cells are different among these mice. The ratio in normal BALB/c mice is $< 1/500$ and that in NZW mice is 1/406. The ratios are significantly higher not only in 10-month-old (1/54) but also in 2-month-old B/WF1 (1/73) mice than normal mice, despite the fact that the latter exhibit no lupus lesions. Although the difference in the ratio between BALB/c and NZW mice seems to remain controversial, these results may indicate that the high frequency of IgG1 anti-DNA antibody-producing cell precursors in lupus mice is not attributed either to the Igh haplotype or to ageing of the tested mice, and the precursor cells for IgG1 anti-DNA antibody-producing cells are present at a high frequency before the disease is developed in lupus-prone mice.

Loss of DNA reactivity after isotype switch to IgG in clonal B cell cultures

To clarify the reason for the difference in the precursor frequency between IgM and IgG1 anti-DNA antibody producers, the reactivity with DNA of IgM and IgG derived from single B cells of BALB/c mice was examined. When spleen cells from 2-month-old female BALB/c mice were cultured at a cell concentration of eight cells per well, less than one antibody-producing cell precursor was given to each well. Therefore, according to Poissonian distribution almost all immunoglobulins detected in these cultures can be considered to be of a single B cell origin. Five independent experiments were carried out under this culture condition, and the results are summarized in Table 2. We obtained 296 IgM-positive clones from more than 1500 cultures, and 14 clones showed reactivity with DNA. The isotype switch to IgG occurred in four out of 14 IgM anti-DNA antibody-producing clones and in 88 out of 282 IgM-producing clones, indicating that the frequencies of isotype switch among

Table 1. Comparison of the ratio of the frequency for anti-DNA antibody-producing cells per total immunoglobulin producing cells between normal and lupus-prone mice

	BALB/c (2 months old)	NZW (2 months old)	B/WF1 (2 months old)	B/WF1 (10 months old)
IgM anti-DNA antibody/IgM	1/13	1/12	1/12	1/14
IgG1 anti-DNA antibody/IgG1	<1/500	1/406	1/73	1/54

Spleen cells from each group of mice were cultured at limiting dilution together with 6×10^5 of nylon wool-passed thymocytes of B10Cr mice in the presence of LPS (60 μg) and IL-4 containing culture supernatant. Immunoglobulin synthesis was measured after 7 days of cultivation by solid-phase RIA. The frequencies were determined by the use of Poissonian analysis.

Table 2. Frequency of isotype switch among IgM-producing cells

Experiment no.	Reactivity with DNA	IgM-producing wells	IgG-producing wells in IgM-producing wells	Ratio
1	+	3	1	0·33
	−	53	12	0·23
2	+	2	1	0·50
	−	49	11	0·22
3	+	2	0	0·00
	−	21	12	0·57
4	+	2	1	0·50
	−	29	8	0·28
5	+	5	1	0·20
	−	130	45	0·35
Total	+	14	4	0·29
	−	282	88	0·31

Spleen cells of 2-month-old BALB/c mice were cultured at a concentration of less than one antibody-producing cell precursor per well with nylon wool purified B10Cr thymocytes in the presence of LPS and IL-4.

Table 3. Radioactive counts (ct/min) of four clones producing IgM anti-DNA antibody and IgG

Clone no.	IgM	IgM anti-DNA antibody	IgG	IgG anti-DNA antibody
1	1338*	100*	246*	7
2	1209*	88*	954*	26
3	3424*	89*	256*	12
4	6097*	189*	513*	30

The four clones produce both IgM anti-DNA antibody and IgG, as shown in Table 2.

* Values show a significant level of antibody production (above the mean counts of background cultures + 3 s.d.).

IgM-producing clones are independent of the antigen specificity.

Anti-DNA antibody of IgG class in the culture supernatants of the four clones which produced both IgM anti-DNA antibody and IgG was assayed, and the results are shown in Table 3 together with the radioactivities for IgM, IgM anti-DNA antibody, and IgG. The IgG derived from these four clones exhibited no anti-DNA activity. Although the amounts of IgG derived from these four clones are lower than those of IgM (Table 3), our preliminary experiments showed that the assay system is sensitive enough to detect the anti-DNA activity of monoclonal IgG anti-DNA antibodies at smaller amounts than those of the four clones. Thus, the results indicated that all of these four clones had lost the reactivity with DNA after isotype switch to IgG.

DISCUSSION

The present study was performed to compare the frequency of precursors for high-affinity anti-DNA antibody, which could react with DNA after isotype switch to IgG-producing cells, in the pre-immune B cell repertoire in normal and lupus mice. Stimulation with LPS and IL-4 *in vitro* preferentially promotes the isotype switch to IgG1 of B cells in the pre-immune repertoire of both normal and lupus mice. It was shown that the number of precursor cells for IgG1 anti-DNA antibody-producers in the pre-immune B cell repertoire of both young and old B/WF1 mice was higher than that in normal young BALB/c and NZW mice. These results indicated that the presence of precursors for IgG1 anti-DNA antibody producers in B/WF1 mice at a higher number was attributable neither to Igh haplotype nor to age of mice examined, and that the precursor cells for IgG1 anti-DNA antibody-producing cells existed before B/WF1 mice exhibited disease manifestation.

Since the B cells of B/WF1 mice are suggested to be activated *in vivo* regardless of the age (Izui, McConahey & Dixon, 1978; Theofilopoulos *et al.*, 1980), it could be that IgG1 anti-DNA antibodies detected in the supernatants of the cultures with LPS and IL-4 were derived from *in vivo* activated B cells. This may not necessarily be true, however, because IgG1 anti-DNA antibody production was induced in the cultures of spleen cells from young B/WF1 mice, in whose sera IgG anti-DNA antibody production was not observed at a similar level to that in the sera of old B/WF1 mice.

The pre-immune B cell repertoire should have been formed by the environmental selection process, including the interaction with self-antigens and the idiotype network, via divalent surface immunoglobulin molecules which are constructed by the assembly of germ line variable region gene segments of heavy and light chains (Klinman, Wylie & Cancro, 1980; Nishikawa,

Takemori & Rajewsky, 1983). We showed in the present study that the IgG antibodies produced by mitogen-activated BALB/c splenic B cells which were originally producing DNA-reactive IgM antibodies were no more reactive with DNA. These results may indicate that the affinity of IgM anti-DNA antibodies produced by mature B cells of normal mice is not high enough to retain the reactivity with DNA after isotype switch to IgG, which results in the reduction of valency without any change in the antigen binding sites. In lupus-prone mice, it has been shown that B cell formation process is activated in the bone marrow of young NZ mice (Jounouchi *et al.*, 1983), and that immature B cells reactive with hapten antigens are not eliminated (Cowdry *et al.*, 1987). We have also found that the avidity of IgM anti-DNA antibodies produced by B/WF1 mature B cells was much higher than that of the antibodies produced by spleen B cells of normal mice (Tsubata *et al.*, 1988), suggesting that old B/WF1 mice are unable to eliminate high-affinity anti-DNA B cells. Thus, it is inferred that the existence of precursors for IgG1 anti-DNA antibody producers in the pre-immune B cell repertoire of young and old B/WF1 mice may be attributed to the defective environmental selection in B/WF1 mice. IgG anti-DNA antibodies observed in B/WF1 mice may be derived from the B cells producing such high affinity IgM anti-DNA antibodies.

Although IgG1 anti-DNA antibody-producing cell precursors exists in young B/WF1 mice, IgG anti-DNA antibody is usually absent in these mice. Therefore, some additional mechanisms may be required to induce the production of IgG anti-DNA antibody. Indeed, the studies on the heavy and light chain variable region sequences of several IgG anti-DNA antibodies derived from lupus-prone mouse strains have shown that variable region genes of IgG anti-DNA antibodies underwent somatic mutations that were accompanied by amino acid replacements (Schlomchik *et al.*, 1987; Dersimonian *et al.*, 1987; Behar & Scharff, 1988; Eilat, Webstar & Rees, 1988). These results strongly suggested that an antigen-driven affinity maturation process is one of the major mechanisms for pathogenic autoantibody production (Eilat *et al.*, 1989). Therefore, two different mechanisms (Klinman, Eisenberg & Steinberg, 1990), the existence of precursors for IgG anti-DNA antibody producers, and the antigen-driven affinity maturation process, may be included in the pathogenic autoantibody production process. Since the level of antigen-driven antibody response is dependent on the number of cells within a diverse B cell repertoire which are selectively stimulated (Klinman, 1972), the high frequency of precursors for IgG anti-DNA antibody-producing cells observed in the pre-immune B cell repertoire of lupus-prone mice may be one of the most crucial factors leading to IgG anti-DNA antibody production.

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