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IgG anti-cardiolipin antibodies in murine lupus

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SUMMARY

The frequency and nature of IgG anti-cardiolipin and anti-ds-DNA antibodies among MRL/lpr, MRL/+ and NZB/W F1 mice (murine lupus strains) and non-autoimmune inbred strains of mice (NIH/Swiss and Balb/c) were analysed by ELISA. High titres of anti-ds-DNA were detected in autoimmune strains (MRL/lpr, 69%; MRL/+, 50%; and NZB/W, 80% positive), whereas anti-cardiolipin antibodies were detected only in MRL/lpr (69%) and MRL/+ (17%) mice. IgG subclass analysis of these antibodies in 20 MRL/lpr sera revealed that all four subclasses were represented. When tested for fine antigenic specificity, anti-cardiolipin antibodies in MRL/lpr and MRL/+ mice bound to acidic phospholipids rather than to neutral phospholipids and were not inhibited by DNA. In MRL/lpr mice, anti-cardiolipin antibodies were first detected at 2 months, peaked around 5 months and then declined preterminally. To determine whether complications associated with anti-cardiolipin antibodies were present in MRL/lpr mice, blood counts were performed and litter sizes were determined. Although no significant decreases in the red and white blood cell counts were observed in MRL/lpr mice, platelet counts were significantly lower compared with NIH/Swiss ($P<0{\cdot}001$) and Balb/c ($P<0{\cdot}005$) mice. MRL/lpr mice had significantly fewer pups per delivery compared with a normal strain (MRL/lpr, $5{\cdot}3+2{\cdot}6$; NIH/Swiss, $7{\cdot}2\pm2{\cdot}1$; $P<0{\cdot}002$). These observations indicate that the serological characteristics of IgG anti-cardiolipin antibodies in MRL/lpr mice are similar to those of anti-cardiolipin antibodies in humans with lupus. Whether these autoantibodies are pathogenetically related to thrombocytopenia and a small litter size in MRL/lpr mice remains to be determined.

Keywords MRL/lpr mice anti-cardiolipin antibodies anti-DNA antibodies thrombocytopenia systemic lupus erythematosus

INTRODUCTION

Anti-cardiolipin antibodies in human systemic lupus erythematosus (SLE) are associated with serious clinical complications such as thrombosis (Harris *et al.*, 1983), intra-uterine fetal death (Derue *et al.*, 1985; Lockshin *et al.*, 1985), and thrombocytopenia (Harris *et al.*, 1985a); these complications are collectively called the 'anti-phospholipid syndrome' (Hughes, Harris & Gharavi, 1986). Although it has been suggested that high titres of IgG anti-cardiolipin antibodies are most strongly associated with these events (Harris *et al.*, 1986; Gharavi *et al.*, 1987), the mechanism(s) by which the antibodies cause clinical complications remain unknown. A major impediment to further studies of the anti-phospholipid syndrome has been the absence of a suitable animal model. Since monoclonal antibodies with anti-phospholipid activity have been produced from the spleens obtained from mice with SLE (Lafer *et al.*, 1981), we undertook a systematic study of IgG anti-cardiolipin antibodies in three lupus strains of mice, MRL/lpr, MRL/+ (Murphy & Roths, 1979) and NZB/NZW F1 (Helyer & Howie, 1963). The results of these studies indicate that all three lupus strains of mice have high titres of anti-DNA antibodies, but that only the MRL/lpr and MRL/+ strains have elevated levels of IgG anti-cardiolipin antibodies.

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MATERIALS AND METHODS

Mice

NZB/NZW F1 and an inbred strain of normal control mice, NIH/Swiss, were bred and maintained at the Hospital for Special Surgery (Mellors *et al.*, 1971). MRL/Mp-lpr/lpr (MRL/lpr) mice were purchased from Jackson Laboratories, Bar Harbor, ME. MRL/lpr/++ (MRL/+) mice were kindly provided by Dr Y. Rosenberg. Balb/c mice were purchased from the Charles River Laboratories, Wilmington, MA. Mice were bled from the retro-orbital plexus and sera were stored at −20°C. Sera from 45 MRL/lpr mice aged 3–5 months (26 female, 19 male); 18 MRL/+ aged 5–16 months (12 female, 6 male); 23 NZB/W mice aged 5–11 months (16 female, 7 male); 21

NIH/Swiss mice aged 6–9 months (14 female, 7 male); and 11 Balb/c mice aged 6 months (all male) were included in the initial study.

ELISA

Murine anti-phospholipid antibodies were detected by an ELISA similar to that described previously for humans (Gharavi *et al.*, 1987). Briefly, ELISA plates were coated with cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidyl ethanolamine, or phosphatidylcholine at a concentration of 50 μg/ml. Cardiolipin was dissolved in ethanol, and other phospholipids were dissolved in methanol chloroform (3:1 v/v). Plates were left to dry overnight at 4°C, then blocked for 2 h with 2% bovine serum albumin (BSA) in 0·15 M saline buffered with 0·01 M phosphate, pH 7·4 (PBS). Mouse sera were diluted 1:100 in 10% adult bovine serum (ABS/PBS) and 50 μl added to each well in duplicate. After 3 h of incubation at room temperature, a 1:1000 dilution of alkaline phosphatase-conjugated anti-mouse IgG (Sigma Chemical Co., St Louis, MO) in 10% ABS was added and the plates were further incubated for 1 h. The reaction was developed with *p*-nitrophenylphosphate in diethanolamine buffer, pH 9·8 and the optical density (OD) was read at 405 nm on a Titertek multiscanner (Flow Laboratories). Antibodies to ds-DNA were detected using similar methods, except that the plates were sequentially coated with 50 μg/ml of poly-L-lysine ($M_r > 300$ kD; Sigma), followed by 100 μg/ml of calf thymus ds-DNA (Sigma) in 0·01 M Tris-EDTA, pH 8·0. The wells were washed and digested with S1-nuclease (BRL, Rockville, MD), 100 U/ml in 0·02 M sodium acetate, 0·05 M NaCl, 0·001 M $ZnCl_2$, 5% glycerol, pH 4·6, for 1 h at 37°C (Gharavi *et al.*, 1988). The rest of the procedure was the same as that for anti-phospholipid antibodies. For both assays, values >4 s.d. above the mean of the value obtained from the NIH/Swiss mice were considered positive.

IgG subclass analysis

Anti-cardiolipin and anti-DNA ELISAs were performed as described above, except that rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Litton Bionetics, Kensington, MD) were used as second antibodies. Each anti-serum was used at a 1:1000 dilution since they produced equal ODs on ELISA plates coated with 1 μg/ml of the corresponding purified monoclonal IgG subclass at this dilution.

Inhibition studies

Sera at the dilutions which gave 50% of maximum activity were pre-incubated with cardiolipin micelles in PBS so that the final concentration of cardiolipin was equal to 300 nM phosphorous. After incubation for 1 h at room temperature and 16 h at 4°C, anti-cardiolipin activity was tested by the ELISA as described above. Samples incubated with PBS alone were used as controls. Percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{OD control} - \text{OD with inhibitor}}{\text{OD Control}} \times 100$$

Haematology

White blood cell, red blood cell, and platelet counts from individual blood samples of 24 MRL/lpr, 18 MRL/+, 12 NIH/Swiss, and 10 Balb/c mice were quantified on an ELT8/WS cell counter (Ortho Diagnostic System, Westwood, MA).

Evaluation of pregnancy outcome

To determine whether litter sizes (number of pups per delivery) were similar in MRL/lpr and a control inbred strain of mice, NIH/Swiss, the number of live pups per successful pregnancy were compared. All female mice in this retrospective study were less than 12 weeks old and were housed in the same room under the same conditions.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test.

RESULTS

Distribution of anti-cardiolipin and anti-ds-DNA in autoimmune mice

We have observed non-specific binding of occasional human sera to ELISA plates that have not been coated with antigen (Levy *et al.*, 1988). To test for non-specific binding in the current study, all mouse sera were screened for binding to ELISA plates treated with ethanol alone—'no antigen plates'. Three MRL/lpr mice and two NZB/W mice sera showed weak binding (OD values, 0·15–0·2) to the plate. However, two NIH/Swiss sera showed very high non-specific binding to the 'no antigen plate' (OD values, 0·7–0·9). These two sera were excluded from the study. The frequencies and levels of anti-cardiolipin antibodies and anti-DNA in the five strains of mice are shown in Fig. 1. The frequency of detection of anti-cardiolipin antibodies (29 out of 45; 64%) and anti-DNA (31 out of 45; 69%) was high in the MRL/lpr mice. Both antibodies were present but were detected with lower frequencies in MRL/+ mice, anti-cardiolipin—three out of 18 (17%); and anti-DNA—nine out of 18 (50%) positive. In contrast, no significant anti-cardiolipin activity was detected in NZB/W F1 sera, despite anti-ds-DNA activity in 16 out of 20 (80%) of samples tested. None of the NIH/Swiss or Balb/c sera showed significant binding to DNA- or cardiolipin-coated plates.

To determine whether all MRL/lpr mice of the same age had similar levels of anti-cardiolipin antibodies, sera from 17 additional MRL/lpr mice, 16 weeks of age (10 female, 7 male), were tested for anti-cardiolipin antibodies by ELISA. Nine (five female and four male) sera were positive (overall frequency, 53%).

Inhibition studies

The specificity of binding of anti-cardiolipin antibodies was also evaluated by pre-incubating test sera with cardiolipin micelles or DNA prior to performing the standard ELISA. Pre-incubation of 11 high titre MRL/lpr sera with cardiolipin resulted in a 81–94% inhibition of binding in nine of the samples (Fig. 2). The two MRL/lpr serum samples with relatively high non-specific binding to the 'no antigen plate' demonstrated only 61 and 66% inhibition.

In another set of experiments, 10 MRL/lpr sera containing both anti-cardiolipin and anti-DNA were pre-incubated with cardiolipin micelles as described above or DNA (final concentration 250 μg/ml). The mean percentage inhibition in the anti-cardiolipin assay was $78{\cdot}3 \pm 13{\cdot}3$ with cardiolipin and <5%

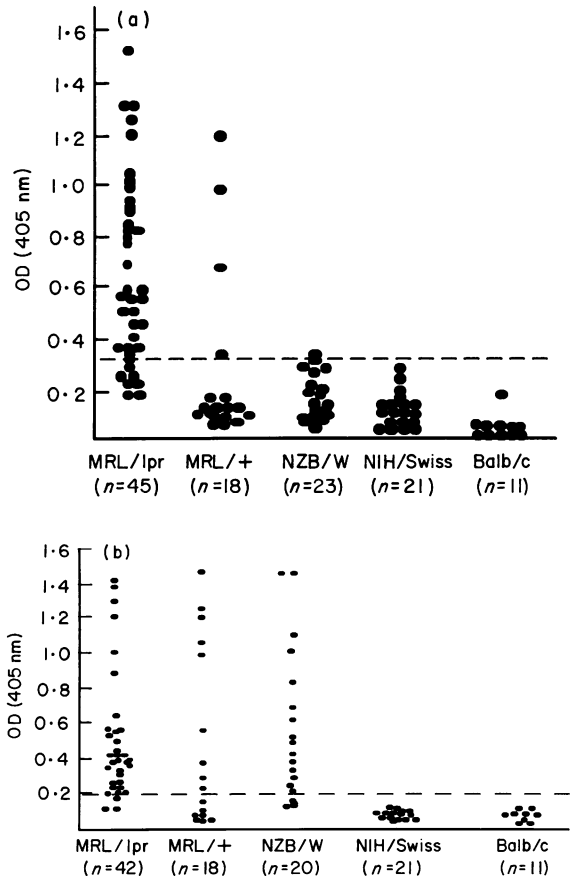

Fig. 1. Levels of anti-cardiolipin (a) and anti-ds-DNA (b) in MRL/lpr, MRL/+, NZB/W, NIH/Swiss and Balb/c strains of mice as measured by ELISA. Sera were tested at a dilution of 1:100 and values >4 s.d. above the mean of the normal controls (indicated by the horizontal lines) were considered positive.

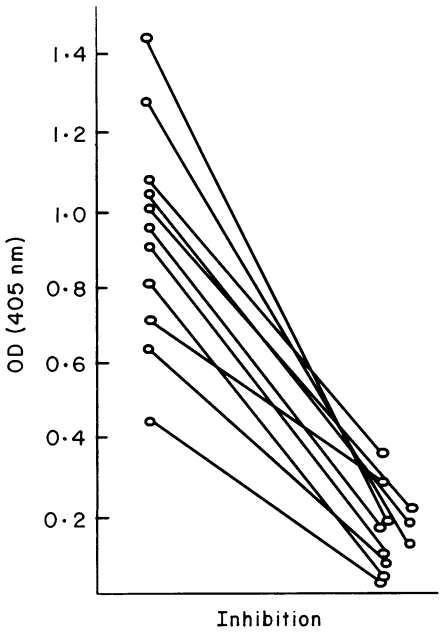

Fig. 2. Inhibition of anti-cardiolipin antibodies by cardiolipin MRL/lpr sera with high titre anti-cardiolipin activity were preincubated with cardiolipin micelles at a final concentration of 300 nM phosphorous prior to assay for anti-cardiolipin activity by ELISA.

with DNA. Conversely, the mean percentage inhibition in the anti-DNA assay was 92·4±6·7 with DNA and <5% with cardiolipin.

Phospholipid specificity of anti-cardiolipin

Sera from four MRL/lpr mice with high titres of anti-cardiolipin activity were tested for binding to phosphatidylserine, phosphatidylinositol, phosphatidyl ethanolamine, and phosphatidyl choline by ELISA. As shown in Fig. 3, IgG antibodies bound to the acidic phospholipids phosphatidylserine and phosphatidylinositol. Binding to phosphatidyl ethanolamine and phosphatidyl choline (neutral phospholipids) was similar to binding to plates treated with organic solvent alone.

Longitudinal studies

Sera from six female and 16 male MRL/lpr mice were collected at monthly intervals from mice aged between 2 and 6 months and tested for anti-cardiolipin activity. The results showed that anti-cardiolipin activity usually became detectable by 2 months of age in females and 3 months in males, was maximal around 5 months, and then declined in the preterminal stage of the disease (6 months of age).

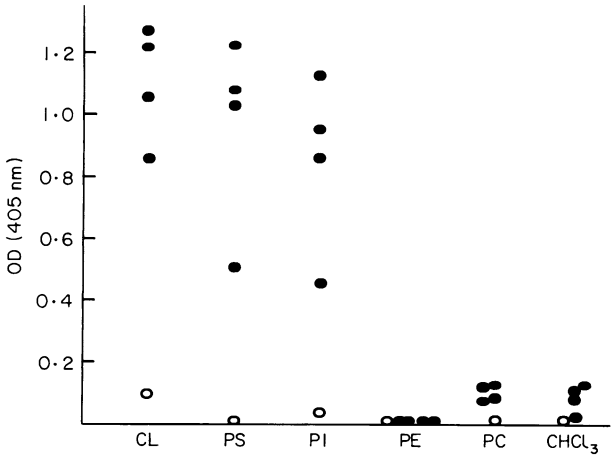

Fig. 3. Phospholipid specificity of MRL/lpr sera. ELISA plates were coated with 50 μg/ml of cardiolipin (CL); phosphatidylserine (PS); phosphatidylinositol (PI); phosphatidyl ethanolamine (PE); phosphatidyl choline (PC); or chloroform-methanol alone ($CHCl_3$). Antibody binding to each coated plate was determined by ELISA with four high-titre MRL/lpr aCL sera (●) and one NIH/Swiss serum (○).

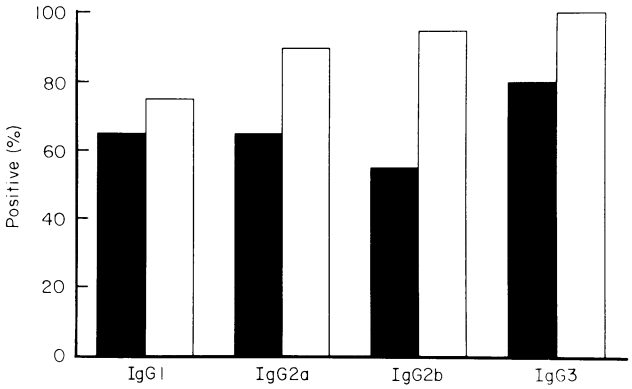

Fig. 4. Frequency of detection of IgG subclasses with either anti-cardiolipin or anti-DNA (□) activity. Sera from 20 MRL/lpr mice positive for IgG anti-cardiolipin antibodies were tested for anti-DNA and anti-cardiolipin antibodies by ELISA as described in Materials and Methods, except that rabbit anti-mouse IgG subclass reagents (diluted 1:1000) were used as second antibodies.

Table 1. White blood cell (WBC), red blood cell (RBC) and platelet counts in MRL/lpr, MRL/+, NIH/Swiss, and Balb/c strains of mice

Strains of mice	WBC ($\times 10^6$/ml)	RBC ($\times 10^6$/ml)	Platelets ($\times 10^6$/ml)
MRL/lpr ($n=24$)	15·22±11·25*	9·11±0·68†	897·2±263·9‡
MRL/+ ($n=18$)	4·3±1·28	9·96±0·442	1251±199·8
NIH/Swiss ($n=12$)	4·66±0·98	9·26±0·66	1463·5±143·8
Balb/c ($n=10$)	9·17±3·44	9·73±1·02	1385±410

* Values significantly higher than MRL/+ ($P<0·001$), NIH/Swiss ($P<0·001$) and Balb/c ($P<0·05$).

† Values not significantly different from control mice.

‡ Values significantly different from MRL/+ ($P<0·001$), NIH/Swiss ($P<0·001$) and Balb/c ($P<0·005$).

IgG subclass

Sera from 20 seropositive MRL/lpr mice were analysed for aCL and a DNA subclass. There was an approximately equal distribution of sublclasses for both autoantibodies (Fig. 4).

Haematology

Leucocyte, erythrocyte, and platelet counts from 24 MRL/lpr, 18 MRL/+, 12 NIH/Swiss and 10 Balb/c mice were determined and compared (Table 1). The leucocyte count was significantly higher in MRL/lpr mice, due to the increased number of lymphocytes. The erythrocyte counts were similar in the four strains, but the platelet counts were significantly lower in MRL/lpr mice compared with MRL/+ ($P<0·001$), NIH/Swiss ($P<0·001$) or Balb/c mice ($P<0·005$). When platelet counts and anti-cardiolipin levels were measured from the same bleed in 20 MRL/lpr and 18 MRL/+ mice, eight out of 12 MRL/lpr and two out of three MRL/+ mice with elevated anti-cardiolipin had relatively low platelet counts ($<1100\times10^6$/ml). However, there was no statistically significant relation between the anti-cardiolipin level and the platelet count ($r=0·24$; $P>0·05$).

Pregnancy outcome

The number of live born pups per pregnancy from 18 breeding pairs of MRL/lpr mice were compared with the number of pups from 18 breeding pairs of the age-matched inbred NIH/Swiss mice. There was a significantly smaller litter size in the MRL/lpr strain (MRL/lpr, 5·3±2·6; NIH/Swiss, 7·2±2·1; $P<0·002$).

DISCUSSION

Although the presence of anti-phospholipid antibodies in autoimmune mice has been described in some reports (Lafer *et al.*, 1981; Rauch *et al.*, 1982; Serban *et al.*, 1985), these antibodies were mostly polyspecific monoclonal IgM antibodies which reacted with DNA, polynucleotides, TNP and phospholipids including cardiolipin. Furthermore, no attention had been paid to the characteristics of polyclonal IgG anti-cardiolipin antibodies and possible clinical associations of these antibodies in mice. In this study, we have evaluated murine anti-cardiolipin antibodies of the IgG isotype which are a better marker for clinical complications in human SLE (Harris *et al.*, 1986; Gharavi *et al.*, 1987), and less likely to be influenced by low-affinity (Smeek *et al.*, 1988) or non-specific (Levy *et al.*, 1988) binding. The use of a 'no antigen plate' and the inhibition studies confirmed that the anti-phospholipid antibodies detected in the sera of MRL/lpr mice are directed against cardiolipin and similar phospholipids. The fine specificity of these murine antibodies (binding to acidic phospholipids such as phosphatidylserine and phosphatidylinositol, but not to neutral phospholipids such as phosphatidyl ethanolamine and phosphatidyl choline) is the same as that observed for human anti-phospholipid antibodies in autoimmune diseases (Harris *et al.*, 1985b). Although the small amount of serum obtained from the retro-orbital plexus precluded testing for anti-coagulant activity, Ichikawa *et al.* (1988) have shown that some monoclonal anti-cardiolipin antibodies obtained from an MRL/lpr mouse had anti-coagulant activity.

Despite the presence of high levels of anti-ds-DNA antibodies in both the MRL/lpr and NZB/W F1 strains of mice, in agreement with previous studies (Theophilopoulos & Dixon, 1985), IgG anti-cardiolipin antibodies were detected only in MRL/lpr sera. This finding, as well as the failure of DNA to inhibit the binding of anti-cardiolipin antibodies underscores the distinctive nature of IgG anti-cardiolipin antibodies (Gharavi *et al.*, 1988; Harris *et al.*, 1985b) and strongly suggests that the majority of polyclonal IgG anti-cardiolipin antibodies and anti-ds-DNA are not cross-reactive. As with several other autoantibodies detected in MRL/lpr sera, such as anti-Sm (Eisenberg, Craven & Cohen, 1987) and anti-ribosome P (Bonfa *et al.*, 1988), antibodies were first detected at the age of 2–3 months, peaked around 4–5 months, and then usually declined when the animals were pre-terminal (5–7 months).

In normal mouse strains, the major IgG subclass responding to foreign protein antigens is IgG1 (Rosenberg & Chiller, 1979), whereas IgG3 responses are dominant in the response to carbohydrate antigens (Perlmutter *et al.*, 1978). Subclass profiles of autoantibodies in MRL/lpr mice show an unusual IgG2a

predominance for anti-Sm (Eisenberg, Winfield & Cohen, 1982) and anti-P (Elkon *et al.*, 1988), but not for anti-DNA antibodies (Eisenberg *et al.*, 1982). In agreement with the latter study, we detected IgG anti-DNA of all subclasses and also observed that anti-cardiolipin antibodies were almost equally represented amongst all subclasses. Although the total serum concentration of IgG3 comprises less than 5% of any other subclass in MRL/lpr mice (Theophilopoulos & Dixon, 1985), the levels of IgG3 anti-cardiolipin antibodies were similar to those of the other IgG subclasses (data not shown). The presence of both complement-fixing and non-complement-fixing anti-cardiolipin antibodies is therefore similar in murine (MRL/lpr) and human SLE (Gharavi *et al.*, 1988). Whether the differences in subclass distribution of autoantibodies against protein *versus* non-protein antigens reflect differences in T cell regulation (Mongini, Stein & Paul, 1981;- McKearn *et al.*, 1982) and whether they relate to possible clinical complications as suggested in human SLE (Gharavi *et al.*, 1988) remains to be determined.

The major focus of interest in anti-phospholipid antibodies in human SLE currently relates to their strong association with thrombosis, fetal death, and thrombocytopenia (Harris *et al.*, 1985a). Although arteriolar lesions in MRL/lpr mice do not usually contain thrombi (Borden *et al.*, 1983), these mice have not been extensively examined for venous occlusion. The detection of significantly lower platelet counts in the MRL/lpr strain as opposed to normal control inbred strains, NIH/Swiss and Balb/c, raises the possibility that anti-cardiolipin antibodies may be associated with these complications in MRL/lpr mice. The lower platelet count in MRL/lpr mice cannot simply be attributed to bone marrow depression, since these mice had a leucocytosis and a normal red blood cell count. In addition, another autoimmune strain, the NZB, had an average platelet count of 1500×10^6/ml (Mellors, 1966), similar to the platelet counts observed in the normal mice in this study. Nevertheless, since an inverse correlation between anti-cardiolipin antibody levels and platelet counts was not observed in our study, anti-platelet antibodies or circulating immune complexes may also have influenced platelet levels in these mice. Another interesting finding was a smaller litter size in MRL/lpr mice, compared with an age-matched control inbred mouse strain. At present we have no evidence that anti-cardiolipin was causally related to the small litter size in MRL/lpr mice or that the small litter size was due to intra-uterine fetal death. Prospective studies of mice with varying levels of anti-cardiolipin antibodies, as well as examination of other murine lupus strains without anti-cardiolipin antibodies should allow a more thorough evaluation of the role of anti-cardiolipin antibodies in pregnancy in MRL/lpr mice.

Although anti-cardiolipin antibodies are associated with clinical complications in human SLE, there is no convincing evidence as yet that the antibodies are directly pathogenic. In the present report, we have shown that anti-cardiolipin antibodies in MRL/lpr mice are similar in most respects to human anti-cardiolipin antibodies and that this mouse strain has a lower mean platelet count and a smaller litter size when compared with normal control mouse strains. As opposed to studies in humans, a cause–effect relation can be tested readily by passive transfer experiments in mice, and the pathological consequences of the transfers can be determined. Hopefully, information gained from such studies in MRL/lpr mice will provide insight into the relationship between anti-cardiolipin antibodies and the anti-phospholipid syndrome in human SLE.

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