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Early onset of autoimmunity in MRL/++ mice following immunization with β_2 glycoprotein I

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SUMMARY

Antiphospholipid antibodies (aPL) are associated with thrombosis, thrombocytopenia and recurrent fetal loss in humans and in some animal models. Immunization with β_2 glycoprotein I (β_2GPI) induced aPL production in normal rabbits and mice. However, the association of these antibodies with disease manifestations remains controversial. To determine whether induction of aPL by β_2GPI immunization in an autoimmune strain of mice (MRL/++) would result in acceleration of clinical and serological autoimmune disease manifestations, three groups of 8-week-old female mice were studied. One group was immunized with β_2GPI, and one with ovalbumin (OVA); the third was not immunized. After two booster injections, sera were analysed for the presence of anticardiolipin (aCL) and anti-DNA by ELISA and anti-nuclear antibody (ANA) by immunofluorescence. Mice were studied for thrombocytopenia, proteinuria, fecundity rates, litter sizes and the development of central nervous system dysfunction. Elevated levels of aCL, anti-DNA and ANA were detected in all β_2GPI-immunized, in three OVA-immunized, and in none of the unimmunized mice. The anti-DNA antibodies were inhibited by CL micelles, suggesting cross-reactivity between aCL and anti-DNA. Platelet counts, fecundity rates and litter size were reduced in β_2GPI-immunized but not in OVA-immunized or unimmunized mice. None of the mice developed neurological dysfunction or significant proteinuria over a 10-week period post-immunization. These findings suggest that β_2GPI immunization induces aPL in MRL/++ mice associated with accelerated autoimmune manifestations resembling the antiphospholipid syndrome.

Keywords antiphospholipid antibodies β_2 glycoprotein I fetal death thrombocytopenia antiphospholipid syndrome

INTRODUCTION

Antiphospholipid antibodies (aPL) have been associated with clinical manifestations such as thrombosis, thrombocytopenia and recurrent fetal loss [1–4]. These antibodies are thought to be directed against an epitope formed by negatively charged phospholipids complexed with β_2 glycoprotein I (β_2GPI). The pathophysiological relationship of aPL to clinical manifestations is not well understood. We were the first to produce aPL in normal rabbits and mice by immunization with heterologous β_2GPI [5]. Other investigators have reproduced these experiments [6–8], but the association of β_2GPI-induced aPL in normal mice with clinical complications remains controversial [7,8].

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MRL/++ mice are a strain of mice genetically predisposed to developing an autoimmune disorder, including anticardiolipin (aCL) autoantibodies and the symptoms of antiphospholipid antibody syndrome (APS) [9], at the age of approximately 12 months [10,11]. A preliminary report [12] suggests that accelerated neurological manifestations begin to appear several months after β_2GPI immunization in MRL/++ mice, but that study did not examine other clinical manifestations associated with aPL. We undertook the present study to attempt to accelerate clinical and serological autoimmune disease manifestations in MRL/++ mice by immunization with β_2GPI.

MATERIALS AND METHODS

Mice

Three groups of nine MRL/++ female mice, aged 8 weeks,

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were included in the study: β_2GPI group, primary immunization with β_2GPI (20 μg/mouse) in Freund's adjuvant; ovalbumin (OVA) group (immunized control group), primary immunization with OVA (20 μg/mouse) in Freund's adjuvant; and unimmunized group, non-immunized control group.

Neurologic evaluation

Postural response. This is a three-point scale (0–2) assessed by lifting the mouse by its tail. Neurologically normal mice exhibit the following responses: hind limb abduction, spreading of the toes, arching of the back and elevation of the head. 0 = a normal response; 1 = curling of toes; 2 = hind limb adduction [13,14].

Placing reflex. This is a three-point scale (0–2) assessed by holding the mouse by its tail and lowering it slowly towards a horizontal surface. Neurologically normal mice extend their forelimbs when the surface is visualized. 0 = a normal response; 1 = forelimb extension only after the nose touches the surface (loss of vision); 2 = forelimb extension only after the head touches the surface (loss of tactile sensation) [13,14].

Grip strength. This is measured by the number of seconds the mouse is able to hang suspended on a stationary bar. A neurologically normal mouse is able to remain suspended for 30 s or longer [13,14].

Balance beam apparatus. This apparatus consists of a narrow rectangular plexiglass crossbar 120 cm long × 0·7 cm wide × 1·2 cm deep, suspended above a table surface and connecting to two plexiglass platforms. The variables measured in this study were the time, in seconds, required to cross the beam, and the number of right hindfoot slips.

Renal function. The presence of proteinuria was assessed using Rapignost sticks.

Serological tests

The mice were bled retro-orbitally at four time points, and the sera were separated by centrifugation and stored at –20°C until tested. The presence of aCL and anti-DNA antibodies was tested by ELISA assay as previously described [9].

Antinuclear antibodies (ANA) were tested by immunofluorescence using commercial slides (BION HEp-2 antigen substrate for Freund's incomplete adjuvant (FIA) test system) with serial dilutions of the sera from 1 : 40 to 1 : 2560.

Inhibition studies. These were performed using cardiolipin micelles as described previously [15]. In brief, 1 mg cardiolipin in 600 μl ethanol and 400 μl chloroform was evaporated under nitrogen and the residue dissolved in 500 μl PBS and vortexed. Test sera were preincubated at final dilutions of 1 : 100–1 : 800 with the cardiolipin micelles overnight at 4°C before testing for aCL and anti-DNA by ELISA, as described above.

Platelet count. This was performed on the final blood samples when mice reached the age of 24 weeks using the Unopette microcollection system (Baxter SP) and a haemocytometer chamber at × 40 magnification on a light microscope.

Mating and pregnancy outcome studies

The mice were mated at 22 weeks and coital vaginal plugs, indicating mating, were counted every morning. Pregnancy was confirmed by vaginal smears: a homogeneous lymphocyte population indicated pregnancy, as opposed to an epithelial cell or a mixed population of cells. The test was repeated on 3 consecutive days. The mice were killed by cervical dislocation on day 15 of pregnancy, the uteri were retrieved and the number of fetuses was counted.

Statistical analysis

A general linear model was fitted to the differences of the log values at time positions 1 and 4. Fisher's exact test was performed to compare the platelet counts, fecundity rates, and number of viable fetuses, as well as the neurological dysfunction. Fisher's exact test was also used to compare the two control groups separately and to compare the study group with the two control groups combined. Fecundity rate and litter size were also evaluated using a linear model that was fitted to the number of viable fetuses for those mice that became pregnant.

RESULTS

After the second booster injection (week 14 of mice age), mean levels of aCL rose in the β_2GPI group (Fig. 1a), whereas in the two other groups they remained significantly lower. Three mice from the OVA group also developed high aCL levels: β_2GPI group, 1·998 ± 0·190 optical density (OD); OVA group, 0·751 ± 0·267 OD; unimmunized group, 0·357 ± 0·054 OD ($P \leqslant 0{\cdot}0001$ comparing the β_2GPI and other two groups combined).

Similarly, after the second booster injection (Fig. 1b), mean anti-DNA levels rose in the β_2GPI (all mice) and OVA groups (primarily due to high anti-DNA levels in the three OVA-immunized mice that also had high aCL levels): β_2GPI group, 0·804 ± 0·078 OD; OVA group, 0·301 ± 0·032 OD; unimmunized, 0·181 ± 0·007 OD; $P \leqslant 0{\cdot}001$ when comparing the β_2GPI and other two groups combined. While no cross-reactivity was noted between aCL and anti-DNA in MRL/*lpr* mice [9], inhibition studies indicated cross-reactivity between aCL and anti-DNA in these MRL/++ mice. Incubation of the sera with CL micelles, in addition to the inhibition of aCL activity by 87–95%, resulted in the inhibition of anti-DNA activity by 76–90% as well.

The ANA titres in all groups rose in an age-dependent manner. The titres rose earlier and to a higher level in the β_2GPI group than in the other groups. The mean ANA titre after the second booster injection (Fig. 1c) was as follows: β_2GPI group, 649 ± 147; OVA group, 262 ± 95; unimmunized group, 244 ± 99. There was no statistically significant difference between the two control groups, but the β_2GPI group was significantly different from the other two groups combined ($P \leqslant 0{\cdot}0086$).

There were no significant neurological status changes in any group. Only trace proteinuria was seen, which remained stable in all three groups (data not shown).

After mating, the pregnancy rate was 55% in the β_2GPI group, 77% in the OVA group and 100% in the unimmunized group. This represents a significant difference between the β_2GPI group and the unimmunized group ($P = 0{\cdot}0412$), but there was no significant difference between the β_2GPI group and the other two groups combined. The litter size, considering only the mice that became pregnant in each group, was as follows: β_2GPI group, 2·6 ± 1·87; OVA group, 6·17 ± 1·71; unimmunized group, 5 ± 1·4 ($P = 0{\cdot}09$ comparing the β_2GPI and other two groups combined). The number of mice with viable fetuses per total number of mice in each group were as

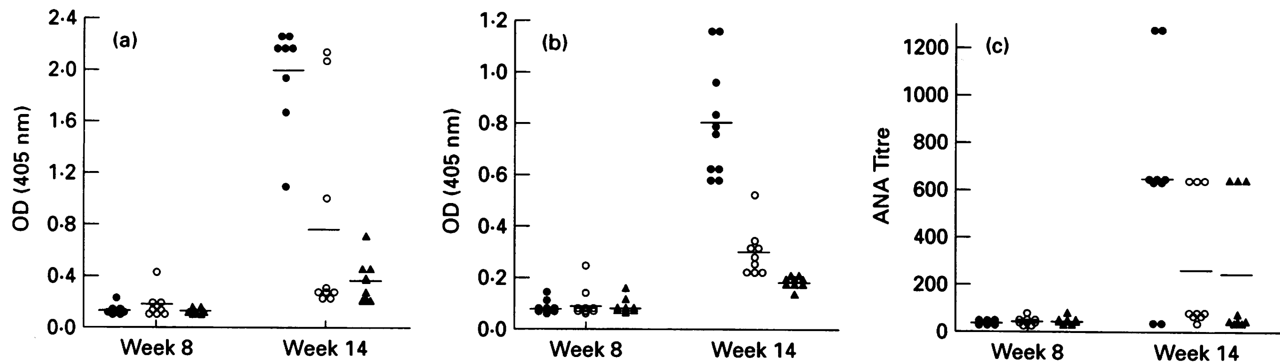

Fig. 1. Anticardiolipin antibody (aCL), anti-DNA, and anti-nuclear (ANA) antibodies. Mouse sera were collected at 8 weeks (pre-immune) and 14 weeks (after second booster injection). Sera were diluted 1:100 in 10% adult bovine serum and tested for aCL (a) and anti-DNA (b) by ELISA. ANA (c) was tested at serial dilutions (1 : 40–1 : 1280) using the Hep-2 cell line as substrate. There were significantly higher levels of aCL, anti-DNA and ANA in mice immunized with β_2 glycoprotein I (β_2GPI) (●) compared with mice immunized with ovalbumin (○) or the non-immunized control group (▲). Fisher's exact test: $P \leqslant 0{\cdot}0001$ for aCL, $P \leqslant 0{\cdot}0086$ for anti-DNA, and $P \leqslant 0{\cdot}05$ for ANA, after the second booster injections.

follows: β_2GPI group, 2 of 9; and other two groups combined, 13 of 18 ($P = 0{\cdot}0194$).

The mean platelet counts at the time of final bleed (24 weeks old) were as follows: β_2GPI, $(1174 \pm 185) \times 10^6$/ml; OVA group, $(1795 \pm 257) \times 10^6$/ml; and unimmunized group, $(1943 \pm 419) \times 10^6$/ml ($P = 0{\cdot}071$ comparing the β_2GPI and other two groups combined).

DISCUSSION

Immunization of MRL/++ mice with β_2GPI induces the production of aCL and other autoantibodies, which are associated with accelerated clinical manifestations characterized by low fecundity rate, small litter size and thrombocytopenia. These clinical manifestations are commonly associated with aPL in humans and in some animal models and, along with thrombosis, define the APS. In previous studies, immunization of both normal and autoimmune mice with monoclonal and polyclonal aPL induced aPL production, which was associated with APS [16,17]. Although accelerated neurological dysfunction has been reported in MRL/++ mice immunized with β_2GPI [12], to our knowledge this is the first report of β_2GPI immunization leading to acceleration of APS in MRL/++ mice. All β_2GPI-immunized mice developed aCL, anti-DNA and ANA antibodies as well as accelerated clinical manifestations. Interestingly, three animals in the OVA-immunized group developed aCL and anti-DNA antibodies, but their presence was not clearly associated with clinical disease manifestations. The pregnancy rate in the OVA-immunized mice was lower than that in unimmunized mice. However, neither post-immunization litter size, the number of viable fetuses per number of pregnant mice, nor platelet count differed between OVA-immunized and unimmunized mice. All of these were significantly lower in β_2GPI-immunized mice compared with the other two groups combined.

A possible explanation for the induction of aCL and anti-DNA in three OVA-immunized mice could be hyperstimulation of the immune system following exposure to Freund's adjuvant or the ovalbumin. This suggests that in this otherwise apparently homogeneous population of mice, there may be those more prone to react to external stimuli with an early autoimmune response. The mycobacterial cell wall components, present in Freund's complete adjuvant (FCA), are more likely to be responsible for the early production of autoantibodies in the three individual mice. Baxter *et al.* reported the precipitation of a systemic lupus erythematosus (SLE)-like syndrome characterized by ANA, anti-DNA, and anti-Sm associated with haemolytic anaemia and renal glomeruli immune complex deposition in non-obese diabetic (NOD) mice by mycobacterial antigen [18]. A single i.v. dose of $2{\cdot}6 \times 10^7$ heat-killed *Mycobacterium bovis* (bacille Calmette–Guérin (BCG)) in 8-week-old autoimmune diabetes-prone NOD mice prevented diabetes but induced a non-organ-specific autoimmune disorder resembling SLE [18]. This is different, however, from the response elicited by immunization with β_2GPI in two important respects. First, an autoimmune response was seen in all β_2GPI-immunized mice, suggesting a more specific role for the β_2GPI in the induction of disease-associated aPL antibodies. Second, clinical disease manifestations were associated with this immune response in β_2GPI-immunized but not OVA-immunized mice, suggesting a difference in pathogenicity of the induced autoantibodies between the two antigens.

In contrast to a prior study, no neurological dysfunction was seen in β_2GPI-immunized mice. Neurological dysfunction was not seen in this prior study until 2 months post-immunization. Therefore, the present study may not have been sufficiently long to detect this manifestation.

MRL/++ mice share over 99% of their genome with another autoimmune strain, the MRL/*lpr* strain. MRL/*lpr* mice develop a variety of autoantibodies, including aPL, as well as clinical manifestations of autoimmunity, including features of APS. These serologic and clinical manifestations usually begin at 12–14 weeks old. In contrast, MRL/++ mice are clinically and serologically normal until 12–14 months of age. Interestingly, the anti-DNA seen in MRL/*lpr* mice are not cross-reactive with aCL, as was seen in the MRL/++ mice [9] in the present study. This suggests that immunization with β_2GPI in MRL/++ mice may lead to a more restricted autoimmune syndrome than is seen in MRL/*lpr* animals, and may be more closely related to production of aCL. Other clinical differences

between β_2GPI-immunized MRL/++ and MRL/*lpr* mice lend further support for this; severe autoimmune-mediated kidney disease and necrotizing vasculitis of the skin, both common features of MRL/*lpr* mice, were not seen in any mice in the present study.

In this study we demonstrated that induction of aPL in an autoimmune-prone strain of mouse also accelerates generalized autoimmunity, expressed by production of a wider range of autoantibodies. In addition, a more restricted autoimmune syndrome, probably related to aPL, was also seen. As such, this model holds great promise for a better understanding of the pathophysiological relationship of aPL to associated disease manifestations, and will be important in testing therapeutic strategies.

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