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Analysis of the genetic encoding of oestradiol suppression of delayed-type hypersensitivity in (NZB × NZW) F_1 mice

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SUMMARY

Oestrogen (E2) has been suggested to be responsible for the female preponderance for systemic lupus erythematosus (SLE) and for exacerbations of disease during pregnancy. In lupus-prone (NZB × NZW) F_1 (NZB/W) mice, sex hormones also influence disease progression, thus long-term treatment of NZB/W mice with high doses of oestradiol increases the mortality in immune-complex mediated disease. We have previously demonstrated that E2 suppression of delayed-type hypersensitivity (DTH) to oxazolone (OXA) in NZB/W mice is inherited from the healthy NZW (H-2^z) and not from the autoimmune NZB (H-2^d) parental strain. In this paper we have analysed the influence of E2 on DTH and antibody responses to OXA in backcrosses of NZB/W mice and the NZB and NZW parental strains. Suppressed DTH was found in 15/16 (94%) of female (NZB/W × NZW) F_1 (NZB/W/W) mice treated with E2. In contrast, only 32/63 (51%) of (NZB/W × NZB)F_1 (NZB/W/B) mice treated with E2 displayed suppressed DTH reactivity. These two findings are compatible with a single, rather than multiple, dominant gene inherited from the NZW strain encoding for E2-mediated suppression of DTH in NZB/W mice. FACS analysis, using a monoclonal antibody recognizing the H-2^z but not the H-2^d locus, identified the H-2 expression (H-2^{dd} and H-2^{dz}) of the NZB/W/B backcrosses and revealed that E2 suppression of DTH is not linked to the H-2 haplotype of the backcrosses. Furthermore, E2 treatment of NZB/W/W mice, but not of NZB/W/B mice, significantly enhanced the serum levels of anti-OXA antibodies of both IgG and IgM classes. Based on our results it is tempting to speculate whether similar genetic factors for E2 sensitivity of the immune system may be of importance for the female predominance of human SLE.

INTRODUCTION

New Zealand Black (NZB) mice spontaneously develop mild glomerulonephritis and production of anti-erythrocyte antibodies, whereas New Zealand White (NZW) mice are phenotypically healthy.[1] The (NZB × NZW) F_1 (NZB/W) hybrid mice spontaneously develop an accelerating autoimmune disease with severe immune complex-mediated glomerulonephritis and excessive production of autoantibodies; thus NZB/W mice constitute a model for human systemic lupus erythematosus (SLE).[1,2]

In SLE, oestrogen has been suggested to be responsible for the female preponderance for disease and exacerbations of disease during pregnancy.[3] In NZB/W mice sex hormones also influence disease progression. Thus, long-term treatment of NZB/W mice with high doses of oestradiol increases the mortality in immune-complex mediated disease and enhances the production of autoantibodies.[4,5]

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We have previously shown that E2 influence on the immune system in mice is genetically linked.[6] In some mouse strains E2 exerts a dichotomous effect on the immune system by suppressing the delayed-type hypersensitivity (DTH) and enhancing the antibody responses, whereas in other strains no such effects could be found.[6] Furthermore, we have demonstrated that E2 suppression of DTH found in the NZW, but not in the NZB, parental strain is dominantly inherited in NZB/W mice.[7]

In this report we have extended the study by analysing the influence of E2 on DTH and antibody responses to oxazolone (OXA) in backcrosses of NZB/W and its parental strains.

MATERIALS AND METHODS

Mice

NZB and NZW mice were originally obtained from Bomholtgård (Bolmholtgård, Denmark). NZB × NZW (NZB/W), (NZB × NZW) F_1 × NZB, (NZB/W/B) and (NZB × NZW) F_1 × NZW, (NZB/W/W) backcrosses were bred and maintained in the animal facilities of the Department of Clinical Immunology, University of Göteborg. A total of 99 NZB/W/B and 30 NZB/W/W mice was analysed. Only female mice were used throughout the study.

Castration and hormone treatment

All mice were castrated at the age of 7–8 weeks. Ovaries were removed after a flank incision. The incisions were closed with metallic clips. The operations were carried out under pentobarbital anaesthesia.

Silastic tubes, 2·5 mm of length, packed with 2·5 mg oestradiol benzoate (Sigma, St Louis, MO) were implanted subcutaneously (s.c.) at the time of castration. Control mice had empty tubes implanted. We have previously demonstrated that subcutaneous implantation of oestradiol-containing siliac tubes (E2 tube) in different mouse strains results in considerably increased 17β-oestradiol serum concentrations, well above physiological levels.[6,7]

One group of NZB/W/B mice was treated with s.c. injections of olive oil containing 3·2 μg of oestradiol twice a week. Such treatment results in serum levels of 17β-oestradiol within the physiological range.[8] Controls received olive oil only.

Castration and start of hormone treatment were performed 2 weeks before sensitization with OXA.

Immunization procedure and registration of DTH reactions

Mice were sensitized by epicutaneous application of 150 μl of a mixture of absolute ethanol and acetone (3:1) containing 3% 4-ethoxymethylene-2-phenyloxazolone (OXA; BDH Chemicals, Poole, Dorset, U.K.) on the shaved abdomen and thorax skin. Seven days after sensitization all mice were challenged by topical application of 15 μl 1% OXA dissolved in olive oil on both sides of the right ear. The left ears were exposed to olive oil only. The thickness of both ears was measured 24 hr after challenge using an Oditest spring caliper (Kröplin, Hessen, Germany), as described previously.[9,10] All challenges and measurements were performed under light pentobarbital anaesthesia. The intensity of the DTH reactions was expressed as (right ear thickness − left ear thickness) $\times 10^{-3}$ cm units.

ELISA

Mice treated with siliac tubes were bled 14 days after sensitization with OXA. All sera were collected individually and stored at −20° until use.

Serum antibody activity against OXA was measured by an enzyme-linked immunosorbent assay (ELISA). OXA conjugated to dog serum albumin (DSA-OXA) in molar ratio 20:1[11] was used as coating antigen. Ninety-six well microplates (Dynatech, Denkendorf, Germany) were coated over night at 4° with DSA-OXA (10 μg/well) dissolved in phosphate-buffered saline (PBS). After washing three times in PBS and blocking with 1% bovine serum albumin (BSA) for 2 hr at 20°, the individual sera were serially diluted in PBS and incubated over night at 4°. After another wash, biotinylated $F(ab')_2$ fragments of goat anti-mouse IgG and IgM antibodies (Jackson Laboratories, Baltimore, PA), diluted in PBS-Tween to a final concentration of 0·3 μg/ml, were incubated for 2 hr at 20°. Peroxidase-conjugated avidin (Dakopatts, Copenhagen, Denmark) was diluted in PBS to a final concentration of 0·5 μg/ml and then incubated for 2 hr at 20°. After another wash the antigen–antibody reaction was quantified by addition of the enzyme substrate, 2.2′-azinodi- (3-ethyl benzthia-zoline sulphonic acid) (Sigma), at a concentration of 0·25% (w/v) in 0·1 M citrate buffer, pH 4·0, containing 0·0075% H_2O_2. The absorbance was recorded after 5 minutes in a Titertek scan at 405 nm. The optical density (OD) values registered were related to the OD values obtained from a reference pool of sera from OXA-sensitized mice. All OD values were converted to anti-OXA-specific arbitrary units (AU) using calibration curves based on the OD obtained from serial dilutions of the reference serum. The calibration curve was constructed using a computer program based on weighted logit–log models.[12,13]

FACS analysis

After bleeding of the mice, spleens were dissected out, teased with forceps and passed through a nylon sieve. The cells were suspended in PBS and centrifuged at 515 *g* for min. The pelleted cells were resuspended for 5 min in Tris-buffered 0·83% ammonium chloride to lyse erythrocytes, followed by two washings in PBS. To identify the H-2 expression, the mononuclear cells were incubated for 45 min with 10 μg/ml of the biotin-labelled monoclonal antibody (mAb) 7-16-7 (a kind gift from Dr Frelinger)[14] in PBS-5% foetal calf serum (FCS). This mAb is produced in BALB/c ($H\text{-}2^d$) mice and is directed against an epitope on the class II molecule that is common for several H-2 haplotypes. After three washings in PBS, cells were incubated for 30 min with 0·5 μg/ml of avidin-FITC (Dako, Dakopatts, Denmark) in PBS and, finally, after several washings in PBS, cell staining was evaluated with a Becton-Dickinson FACS analyser.

In initial experiments the efficacy of the mAb was controlled by using spleen cells from NZB ($H\text{-}2^d$), NZW ($H\text{-}2^z$) and NZB/W ($H\text{-}2^{dz}$) mice. The results showed clearly that this mAb was able to identify cells expressing $H\text{-}2^z$ (i.e. cells from NZW and NZB/W) and also that it did not stain cells expressing only $H\text{-}2^d$ (i.e. cells from NZB). Thus, this mAb provided us with the possibility of identifying which of the NZB/W/B mice expressed $H\text{-}2^{dz}$ and $H\text{-}2^{dd}$, respectively. Ninety-nine female NZB/W/B mice were analysed and $H\text{-}2^{dz}$ and $H\text{-}2^{dd}$ were expressed in 51 and 48 mice, respectively.

Statistical analysis

The levels of significance between means of the groups were obtained by Student's two-tailed *t*-test.

RESULTS

Suppression of DTH by E2 is encoded by a single dominant gene

Oophorectomized and empty tube-treated NZB/W/W and NZB/W/B mice, sensitized and challenged with OXA, showed considerable DTH reactivity, as expressed by increase in ear thickness (mean ± SD, 24·7 ± 3·4 and 27·1 ± 4·0 cm $\times 10^{-3}$, respectively). In contrast, oophorectomized and E2-treated NZB/W/W and NZB/W/B mice displayed suppressed DTH reactivity (9·3 ± 4·4, $P < 0{\cdot}001$, and 15·6 ± 6·9 cm $\times 10^{-3}$, $P < 0{\cdot}001$, respectively), compared to empty tube-treated controls (Fig. 1a,b).

Significant suppression of DTH reactivity in E2-treated mice was defined as an increase of ear thickness below 3 SD compared to the mean of control mice. Figure 1a shows that significantly suppressed DTH reactivity was found in 15/16 (94%) NZB/W/W mice implanted with E2-containing tubes. The corresponding numbers for NZB/W/B mice were 11/23 (48%) (Fig. 1b). These frequencies are compatible with the pattern of inheritance of a single dominant gene, originating from the NZW strain, encoding for E2-mediated suppression of DTH.

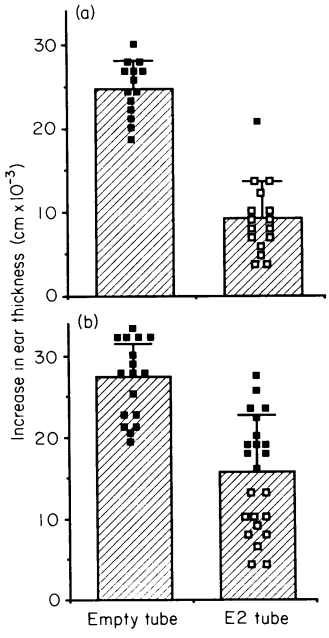

Figure 1. The influence of oestradiol (E2 tube) on DTH responses (mean ± SD) in oophorectomized NZB/W/W (a) and NZB/W/B (b) mice. Each dot represents one mouse. (■) Non-suppressed DTH response. (□) Suppressed DTH response, defined as reactivity below 3 SD compared with mean increase of ear thickness in control mice.

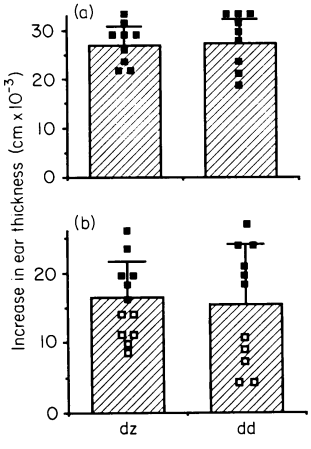

Figure 2. The influence of the H-2 haplotype on DTH responses in oophorectomized NZB/W/B mice treated with empty tubes (a) and E2-containing tubes (b). (■) Non-suppressed DTH response. (□) Suppressed DTH response, defined as reactivity below 3 SD compared with mean increase of ear thickness in control mice.

Lack of correlation between suppression of DTH by E2 and the H-2 haplotype

Spleen cells from NZB/W/B mice were analysed for the expression of the H-2^z haplotype. In Fig. 2a it is shown that in empty tube-treated NZB/W/B mice the H-2 haplotype did not influence the DTH reactivity. Figure 2b shows that in the E2-treated group the number of mice expressing H-2^{dz} and H-2^{dd} was 12 and 11, respectively. The DTH reactivity was equally

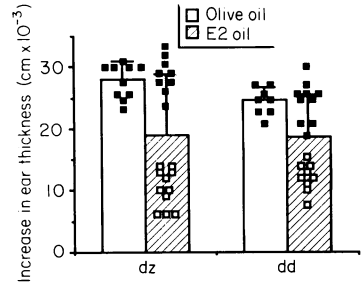

Figure 3. The influence of the H-2 haplotype on DTH responses in oophorectomized NZB/W/B mice treated with 3·2 μg E2 twice a week. Controls received olive oil. (■) Non-suppressed DTH response. (□) Suppressed DTH response, defined as reactivity below 3 SD compared with mean increase of ear thickness in control mice.

Table 1. The influence of E2 treatment on IgG and IgM anti-OXA antibody responses in oophorectomized NZB/W/W and NZB/W/B mice

Strain	Treatment	*n*	Anti-OXA IgG (AU)	Anti-OXA IgM (AU)
NZB/W/W	E2 tube	16	359 ± 112 $P < 0.01$	52 ± 15 $P < 0.001$
	Empty tube	14	241 ± 115	27 ± 8
NZB/W/B	E2 tube	23	292 ± 127 NS	75 ± 24 NS
	Empty tube	22	256 ± 142	97 ± 66

Results are expressed as ELISA arbitrary units (AU) (mean ± SD). NS, not signitificant.

pronounced in E2-treated NZB/W/B mice, with haplotype H-2^{dz} and H-2^{dd} 16·5 ± 5·1 and 15·3 ± 8·5 cm × 10^{-3}, respectively. Furthermore, Fig. 2b shows that 6/12 (50%) of dz- and 5/11 (46%) of dd-expressing NZB/W/B mice displayed significantly suppressed DTH reactivity compared to empty tube-treated controls. The latter finding indicates that the gene encoding for E2 suppression of DTH is not associated with the H-2 complex of NZW mice.

When analysing the influence of physiological doses of E2 (injection of 3·2 μg twice a week) on DTH reactivity to OXA in NZB/W/B mice, similar results were obtained as for mice treated with E2-containing siliac tubes (Fig. 3).

E2 enhancement of anti-OXA antibody responses

Levels of IgG and IgM anti-OXA antibodies were assessed in serum from mice treated with a high dose of E2 and collected 2 weeks after sensitization with OXA. Table 1 shows that NZB/W/W mice treated with E2 tubes displayed significantly increased levels of antibodies to OXA of both IgG and IgM classes ($P < 0.01$ and $P < 0.001$, respectively). In contrast, E2 treatment of NZB/W/B did not significantly influence the IgG and IgM antibody responses to OXA. In addition, there was no difference in anti-OXA antibody responses in NZB/W/B mice expressing H-2^{dd} and H-2^{dz}, respectively (data not shown).

DISCUSSION

We have analysed the genetic background for E2 influence on T- and B-cell immune responses in autoimmune NZB/W mice. In a recently published paper we demonstrated that E2 exerts a dichotomous effect on the immune system of NZB/W mice. Thus, administration of E2 suppressed T-cell reactivity, whereas it enhanced B-cell responses.[7] It was also demonstrated that suppression of DTH in NZB/W mice is inherited from the healthy NZW parental strain. However, the E2-mediated enhancing effect on antigen-specific antibody responses was not found in any of the parental strains.[7]

The present results show that E2 suppression of DTH in backcrosses of NZB/W and NZB mice is inherited as a single dominant gene originating from the NZW strain. By determination of the H-2 phenotype of the backcrosses it can also be stated that the gene, encoding for E2-mediated suppression of DTH, is not linked to the H-2 complex. E2-mediated stimulation of antibody production was found only in backcrosses with NZW mice and not the NZB mice, indicating that the gene(s) from the NZW parental strain is also of importance for the E2-mediated enhancing effect on B-cell responses.

It has been suggested recently that the major NZW contribution to the autoimmune disease of NZB/W mice is a dominant gene localized in the H-2 complex and encoding for the reduced production of tumour necrosis factor (TNF-α) in NZW mice.[15,16] In addition, by comparing disease progression in NZB/W mice and in F_1 hybrids of NZB and congenic NZW H-2^d (ZWD/8) mice, Hirose *et al.*[17] demonstrated that an H-2-linked gene(s) from the NZW strain contributes to the autoimmune disease of NZB/W mice. These three studies showing H-2-linked contribution of NZW genes to the disease of NZB/W mice were based on experiments using only female mice. Thus, the accelerated disease in female NZB/W mice compared with males has yet to be clarified.

The influence of sex hormones on the immune system may have significant impact on the development and progression of the lupus-like disease in NZB/W mice. Indeed, female mice are more prone to develop lupus disease than males, resulting in a considerably shortened life span.[18] Furthermore, administration of high doses of E2 to castrated NZB/W mice increased mortality as well as autoantibody formation and aggravated glomerulonephritis.[4,5] Interestingly, such correlation between gender and sex hormones on the one hand and the development and progression of the autoimmune disease on the other has not been demonstrated in the NZB parental strain.[1,4,5] Thus, the mean survival time for female and male NZB mice is similar.[18] The discrepancy between effects of sex hormones on autoimmunity in NZB and NZB/W mice, respectively, has yet to be elucidated. Our results from this and a previous study[7] indicate that the NZW parental strain contributes to the lupus disease of NZB/W mice with a single dominant gene encoding for E2-mediated suppression of T-cell reactivity and with a gene(s) promoting E2-mediated enhancement of B-cell responses. Studies concerning the relation between E2 sensitivity of the immune system and the progression of the autoimmune disease in NZB/W/B mice are underway.

The sex ratio for the incidence of adult human SLE has been reported to be 13 to 1 for female and male, respectively.[19] Based on our results in the NZB/W model for SLE it is tempting to speculate whether similar genetic factors for E2 sensitivity of the immune system may be of importance for the female predominance of human SLE and for exacerbations during pregnancy.

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