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Functional and quantitative changes of immune cells of ageing NZB mice treated with nandrolone decanoate. I. Effect on survival and autoantibody development

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SUMMARY

We analysed the effect of nandrolone decanoate (ND) on functional and quantitative changes in immune cell populations, on survival, and on autoantibody production of female New Zealand Black (NZB) mice. Our results confirmed that, with increasing age, untreated NZB mice display a lower natural killer (NK) cell activity, an impaired T-cell function as evidenced by a reduced mitogen lymphoproliferative response, IL-2 production and generation of cytotoxic lymphocytes, a lower level of thymic serum factor (TSF), a reduced percentage of Thy-1$^+$ cells; we also observed an increased incidence of mice with abnormally high levels of anti-DNA in the serum. In addition, we demonstrated an important defect in the IL-1 production by LPS-stimulated macrophages. ND administered to female NZB mice increased the survival time of the animals and reduced the anti-DNA titres. This favourable effect was associated with improved immune responses, especially those mediated by T cells; these included increased IL-2 production, complete recovery of cytotoxic T lymphocytes (CTL), a significant augmentation of the percentage of Lyt-2$^+$ cells and enhanced TSF level. Moreover IL-1 production by macrophages returned to normal. These results suggest that ND acts on T-cell differentiation, either by a direct effect on thymic epithelial cells resulting in an increased TSF release, and/or via macrophage regulatory activity. The protective effect of ND may also be attributed in part to the higher number of Lyt-2$^+$ (suppressor) T cells present in the spleen after treatment.

Keywords autoimmunity androgen cell-mediated immunity

INTRODUCTION

New Zealand Black (NZB) mice developed a spontaneous autoimmune disease and displayed multiple abnormalities in both T and B cell compartments (Steinberg *et al.*, 1981). An early increase in helper function (Evans, Williamson & Irvine, 1968) and decrease in suppressor function mediated by T cells (Cantor *et al.*, 1978) could both contribute to the elevation of autoantibody production. The protective influence of androgens, which were shown to delay autoantibody formation and to prolong survival in experimental models (Roubinian *et al.*, 1978; Steinberg, Smathers & Boegel, 1980), is attributed to its capacity to generate suppressor cells (Weinstein & Berkovich, 1981). Since numerous studies have stressed the profound effect of sex hormones on the thymus (Barr *et al.*, 1984;

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Weinstein & Berkovich, 1981) we were interested in the present paper in studying the changes in T cell and macrophage responses induced by androgen treatment of NZB female mice and therefore in analysing their mechanism leading to decreased or delayed autoimmune manifestations. For this purpose, an anabolic steroid displaying attenuated virilising activity, nandrolone decanoate (ND) administered chronically, was selected because of its previously reported beneficial effects on the survival of (NZB × NZW) F1 mice (Verheul *et al.*, 1981). We found that ND improves survival of NZB female mice, decreases the level of anti-DNA antibodies and modulates their immune system.

MATERIALS AND METHODS

Animals. Two-month old NZB female mice were obtained from Bom Holtgaard Laboratories (Denmark).

Drug treatment and experimental groups. 19-Nortestosterone decanoate (nandrolone decanoate, ND) dissolved in arachis oil was injected subcutaneously in one group of mice every 3 weeks at a dose of 40 mg/kg. Arachis oil alone was injected in control animals according to the same protocol (placebo group). Treatment started at 10 weeks of age and continued until the animals died. Forty mice were included in each group from which 22 were used for assessment of mortality. The following tests were performed on pools of spleen cells, each pool being derived from four animals of the same treatment group at various ages (9, 12, & 15 months). At each time, we used a control pool of spleen cells obtained from four untreated 3 month old NZB mice (considered as young mice).

Natural killer cell activity. Spleen cells at various concentrations from treated and untreated mice were incubated with 10^4 ^{51}Cr-labelled YAC lymphoma target cells for 4 h at 37°C in round-bottomed, 96-well plastic microtitre plates. After incubation, the radioactivity released in 0.1 m of supernatant was measured in a gamma scintillation counter. Cultures were done in triplicate, and the percentage of specific lysis was calculated as follows:

$$\text{Cytotoxicity (\%)} = \left[\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}}\right] \times 100$$

Spontaneous release was obtained in the presence of thymus cells as negative effectors.

Generation of cytotoxic T lymphocytes (CTL). 5×10^6 spleen cells from treated and untreated NZB mice (H-2^d) per well of multiwell plate (Linbro, Flow Laboratories, Rockville, USA) were incubated in the presence or absence of 5×10^4 mitomycin-C-treated EL4 tumour cells (H-2^b). The RPMI 1640 culture medium contained antibiotics, glutamine and 5×10^{-5}M 2-mercaptoethanol (2-ME). After 5 days of culture, the cells were harvested and tested for their cytolytic activity. We mixed 10^4 ^{51}Cr-labelled EL4 target tumour cells with varying numbers of spleen cells for 4 h and the radioactivity was measured in the supernatants. Cultures were done in triplicate. The percentage of specific lysis was calculated as for natural killer (NK) cell actvity, but in this experiment spontaneous release was obtained from lysis of cultures containing 10^4 ^{51}Cr-labelled EL4 cells as targets, together with various numbers of spleen cells cultured in the absence of EL4 tumour cells.

Interleukin 2 (IL-2) production and activity. Spleen cells from treated or untreated NZB mice (5×10^6 cells/ml) were incubated for 24 h with Concanavalin A (Con A) (2 μg/ml), and supernatants were tested for IL-2 activity by measurement of their capacity to induce cytotoxic thymocytes. Thymocytes from C57BL/6 mice (10^6 cells/well) were stimulated with irradiated DBA/2 splenocytes (10^6 cells/well) in the presence or absence of the supernatants at various dilutions. Four days later, 10^4 ^{51}Cr-labelled P815 target cells were added and chromium release was measured in the supernatants after 4 h of incubation.

Characterization of T-cell subsets by use of antisera against surface markers. The percentage of spleen cells expressing Thy or Lyt components was estimated from the lytic effect of spleen cells after exposure to antisera (1:200 dilution for monoclonal anti-Thy 1·2, 1:30 dilution for anti-Lyt 1·2 and anti-Lyt 2·2 alloantiserum, Cedarlane) with low-tox rabbit complement (1:18 dilution, Cedarlane), as described by Cantor and Boyse (1975). Complement and antiserum controls were included. Treatment with anti-Thy 1·2 was performed on the total splenic population and treatment with anti-

Lyt antisera was performed on nylon-non-adherent enriched T-cells prepared according to the method of Julius *et al.* (1973). Results were expressed as the mean for three different experiments; in each experiment, the spleens of two mice per group were pooled.

Interleukin 1 (IL-1) production and activity. Monolayers of approximately 10^5 peritoneal adherent cells/2 ml wells estimated by neutral red uptake were incubated for 24 h in RPMI 1640 medium plus antibiotics with or without the addition of 100 μg of lipopolysaccharide (LPS) from *E. coli* (Difco Laboratories, Osi, Paris, France). Supernatants were discarded, and adherent cells were restimulated with 100 μg of LPS for another 24 h. Cell free supernatants were diluted and added to C3H/HeJ thymocytes (10^7 cells/ml) stimulated with 1 μg of phytohaemagglutinin (PHA). After 3 days of incubation at 37°C, 1 μCi of ^{3}H-thymidine per well was added for the final 4 h. Thymocyte proliferation was expressed as the mean ^{3}H-thymidine incorporation in triplicate cultures.

To test for the presence of thymic serum factor (TSF) and anti-DNA antibodies serum was collected from animals, either during the treatment course when they looked sick or when they were killed for determination of immunological activity.

Evaluation of thymic serum factor. The amount of TSF was measured according to the method of Savino, Dardenne and Bach (1983). Briefly, serum samples were tested at various dilutions for their capacity to confer sensitivity to azathioprine on spontaneous rosette-forming cells.

Assessment for anti-DNA antibodies. A sample (50 μl) from each individual decomplemented serum was mixed with 10 μl of ^{14}C-labelled DNA extract (specific activity, 1814 ct/min) from *E. coli.* The sample contained 5·84 μg/ml of sodium chloride (specific activity, 15–20 μCi/mg, Amersham, Les Ulis, France) and was adjusted to 100 μl with borate-sodium chloride buffer (0·15 M, pH 8·0). The mixture was incubated at 37°C for 30 min and then at 4°C for 3 h. The antigen-antibody mixture was then diluted with buffer and passed over a cellulose ester filter under suction. The filters were washed twice with 10 ml or more of buffer; the filters were dried, placed in counting vials, and covered with plasmagel-toluene scintillation medium. Radioactivity was determined in a beta counter. The results were expressed in terms of ct/min retained on the filter (Attias, Sylvester & Talal, 1973).

Statistical evaluation. Student-Fisher's *t* test and Wilcoxon test were used for determination of the significance of differences between groups.

RESULTS

The immune reactivity of ND-treated and placebo-treated groups was compared with that of young untreated (2 to 3 months old) female NZB mice.

Effect of ND on NK cell activity and on the generation of CTL

Both NK and CTL activities are depressed in 9-month old and in older placebo-treated NZB mice (Table 1). Administration of ND increases significantly NK cytotoxicity and completely restores the defective CTL response of 9 and 12-month old treated mice (Table 1). However, both activities are no more stimulated by the treatment in 15-month old mice and return to values observed in placebo-treated mice.

Effect of ND on IL-2 production

NZB spleen cells stimulated for 24 h with Con A released factors (usually designed as IL-2) which induce thymocytes to differentiate into cytotoxic cells after allogeneic stimulation. Production of IL-2-like activity by cells from 14- to 15-month old placebo-treated NZB mice was significantly reduced compared to that of cells from 2- to 3-month old animals at all dilutions (Table 2). Treatment with ND increased IL-2 production by lymphocytes (significantly for 1/8 and 1/16 dilutions), suggesting that ND enhances helper T-cell activity.

Effect of ND on T-cell markers in the spleen

The percentage of Thy-1 positive cells was significantly reduced ($P < 0·02$) in the spleens of 14-month old placebo-treated NZB mice, whereas the percentage of Lyt-1$^+$ and Lyt-2$^+$ T-cell subclasses in the nylon-non-adherent cell population that had not changed significantly in old-

Table 1. Effect of ND treatment on natural killer and cytotoxic T cell activities

	^{51}Cr labelled target used:	Percentage of ^{51}Cr release ($\pm$s.d.) in the presence of splenic effectors from mice of the following ages: 9 months	12 months	15 months
Placebo treated	YAC*	9·2±0·8‡	4·6±0·4‡	2·1±0·4
NZB mice	EL_4†	3·4±1·2‡	6·8±2·6‡	2·6±2·3
ND-treated	YAC	15·0±1·2§	25·1±0·8§	4·0±0·5
NZB mice	EL_4	16·4±2·0§	20·2±1·4§	2·3±2·5
3-month old	YAC	32·1±2·0	31·4±1·4	26·0±1·9
NZB controls	EL_4	16·8±1·9	23·2±2·5	19·4±1·1

* NK cell assay; results shown for effector:target ratio of 100:1.

† CTL assay; results shown for effector:target ratio of 50:1.

‡ Significantly ($P<0{\cdot}01$) decreased compared to young NZB controls.

§ Significantly ($P<0{\cdot}01$) increased compared to age-matched placebo-treated NZB mice.

Table 2. Level of IL-2-like activity, after Con A stimulation, in the supernatants of spleen cells from placebo-treated and ND-treated NZB mice

Supernatants* derived from Con A-stimulated spleen cells of:	Specific cytotoxicity (%) (±†s.d.) in the presence of supernatants at the following dilutions: 1/4	1/8	1/16
Untreated young NZB mice (2 months)	36·4±7·8	33·8±12·2	24·3±7·4
Placebo-treated NZB mice (14 months)	20·1±5·3‡	4·6±5·2‡	1·5±2·6‡
ND-treated NZB mice (14 months)	17·0±6·1	14·9±0·9§	26·1±4·2§

* Supernatants were produced by spleen cells stimulated for 24 h by 2 μg/ml of Con A.

† IL-2 activity is tested by its capacity to render B6 thymocytes (10^6/well) specifically cytotoxic after 5-day allogeneic stimulation with irradiated DBA/2 cells (10^6/well).

‡ Significantly ($P<0{\cdot}05$) decreased compared to young NZB controls.

§ Significantly ($P<0{\cdot}05$) increased compared to placebo-treated mice.

placebo-treated animals (Table 3). Treatment with ND did not modify the percentage of Thy-1$^+$ and Lyt-1$^+$ cells; only a slight increase in the latter population was observed (69·4% versus 53·6% in untreated aged NZB, $P=0{\cdot}07$, Table 2). However, the percentage of Lyt-2$^+$ cells increased significantly in ND-treated mice (33·7%) compared to that of both aged placebo-treated (19·6%, $P=0{\cdot}05$) and young untreated NZB mice (11·6%, $P<0{\cdot}01$).

Since a macrophage defect might alter T-cell functions we looked at the IL-1 secreting capacity of peritoneal macrophages from placebo-treated and ND-treated NZB mice.

Effect of ND on IL-1 production by macrophages

IL-1 activity, as measured by the capacity to induce proliferation of thymic cells under PHA stimulation, was reduced considerably in the supernatants of aged macrophages from placebo-

Table 3. Distribution of T-cell surface markers among spleen cell populations

Group of NZB mice	Percentage of positive cells in the spleen* (±s.d.) expressing the following markers: Thy-1⁺ cells (Cells among total spleen cell population, %)	Lyt-1⁺ cells (Cells among nylon-non-adherent cells, %)	Lyt-2⁺ cells (Cells among nylon-non-adherent cells, %)
Untreated young (2–3 months)	57·0±4·0 ($P<0.02$)	61·1±6·5	11·6±2·6 ($P<0.01$)
Placebo-treated aged (14–15 months)	44·8±3·2 ($P<0.02$)	53·6±9·6 ($P<0.02$)	19·6±6·0 ($P<0.01$)
ND-treated aged (14–15 months)	49·8±2·5	69·4±5·2 ($P<0.02$)	33·7±6·2 ($P<0.01$)

* Results are means from three different experiments.

treated mice, stimulated with 100 μg of LPS (Table 4). The quantity of IL-1 produced by macrophages of 14-month old ND-treated mice was comparable to that obtained with young untreated macrophages (Table 4).

Effect of ND on TSF levels

The mean TSF titres (expressed in terms of reciprocal dilutions) of sera tested in individuals of placebo-treated group declines sharply in 9- to 11-month old mice (Fig. 1). This decrease in TSF level observed with aging in NZB mice is less pronounced in the group of mice treated with ND and the titres are significantly augmented by the treatment in 9- to 11-month old mice and in mice older than 15 months (Fig. 1).

Table 4. Level of IL-1 activity after LPS stimulation in the supernatant of macrophages from placebo-treated or ND-treated NZB mice

Supernatants derived from LPS-stimulated macrophages† of:	Dose of LPS (μg)	Thymidine incorporation* (ct/min±s.d.) in the presence of supernatants at the following dilutions: 1/4	1/8	1/16
Untreated young	0	532±101	331±84	329±27
NZB mice (2 months)	100	4716±1172	3524±381	1784±470
Placebo-treated	0	1736±426	1335±278	413±107
NZB mice (14 months)	100	2144±157‡	749±180‡	432±131‡
ND-treated NZB mice	0	1274±322	551±144	286±86
(14 months)	100	4891±1222§	4061±1205§	1694±435§
Unstimulated C3H/He thymus cells	0	62±12		
C3H/He thymus cells +PHA	0	231±96		

* Il-1 activity was tested by its capacity to induce proliferation of C3H/He thymocytes stimulated for 48 h by 1 μg PHA.

† Supernatants were produced by peritoneal macrophages stimulated with 100 μg LPS during the second 48 h of culture.

‡ Significantly ($P<0.01$) decreased compared to young NZB controls.

§ Significantly ($P<0.01$) increased compared to placebo-treated NZB mice.

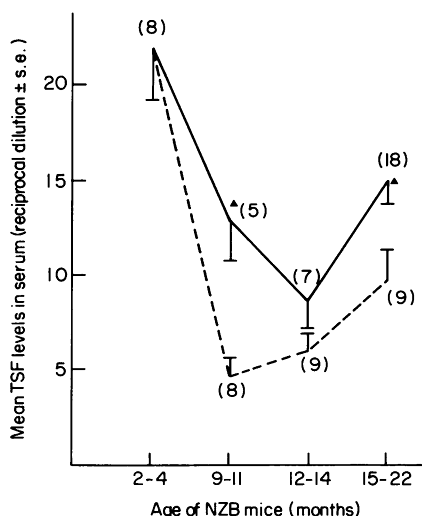

Fig. 1. Mean TSF levels in serum of individual NZB mice (expressed in terms of reciprocal dilutions ± s.e.) as a function of age and treatment: placebo-treated mice (- - -) or ND-treated (——) mice. (*n*) Number of mice in each age group. $P < 0{\cdot}05$ compared to controls (▲).

Effect of ND on the level of anti-DNA antibodies

Sera from 18 young untreated, 27 placebo-treated and 30 ND-treated NZB mice were assessed for the presence of anti-DNA antibodies as measured by the radioactivity entrapped in DNA-anti-DNA complexes (Fig. 2). The incorporation (ct/min ± s.e.) was significantly increased in 9-month old and older placebo-treated NZB mice. ND treatment efficiently reduced the anti-DNA level and a significant difference compared to placebo-treated mice appeared in mice older than 12 months after approximately 9 months of treatment. The 18 ND-treated mice which survived longer than 15 months of age exhibit levels of anti-DNA near to those observed in young untreated mice.

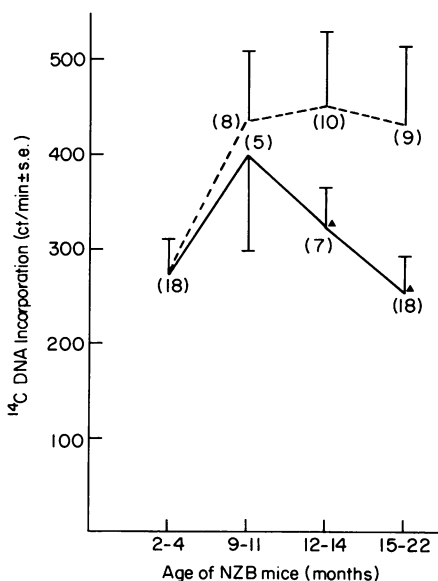

Fig. 2. Level of antibodies binding ^{14}C-DNA (mean ct/min ± s.e.) as detected by the cellulose ester filter radioimmunoassay in the serum of individual NZB mice as a function of age and treatment: placebo-treated mice (- - -) and ND-treated mice (——), (radioactivity added 1814 ct/min). (*n*) Number of mice in each age group. $P < 0{\cdot}05$ compared to controls (▲).

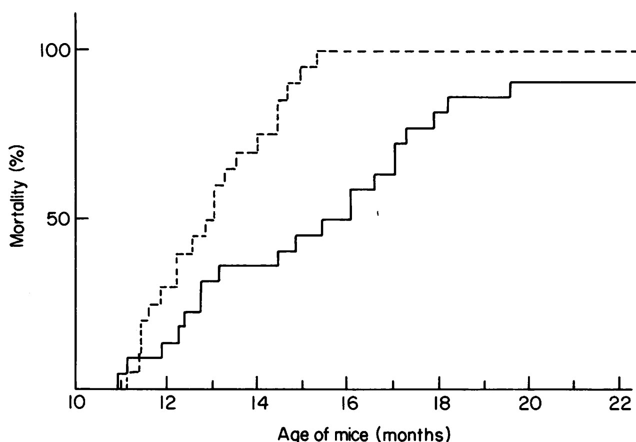

Fig. 3. Effect of repeated i.p. administration of ND (40 mg/kg) on cumulative mortality rate of female NZB mice. The mice were treated every 21 days from 10 weeks of age either with placebo (– – –) or with ND (——).

Effect of ND on survival

The results presented in Fig. 3 demonstrate that ND reduced significantly ($P<0{\cdot}01$ using the Wilcoxon non-parametric test) the mortality rate: 45% of the treated animals (10/22) had died at 15 months of age, versus 95% of the control animals (19/20). Two mice were excluded in the control group because of the presence of an otitis. After treatment, the median survival was increased by 10 weeks (65 versus 55 weeks).

DISCUSSION

This study shows a favourable effect of ND treatment in increasing the survival time of female NZB mice, in effectively reducing the anti-DNA level, and in improving various T-cell and macrophage functions.

Abnormalities of the immune system, atrophy of the thymus (De Vries & Hijman, 1966), decreased level of circulating thymic hormones produced by epithelial cells of the thymus (Savino, Dardenne & Bach, 1983; Bach, Dardenne & Salomon, 1973) high helper activity and deficiency in both number and function in the Lyt 123^+ T cell suppressor subset, are already evident in young NZB mice. Later in life, cell-mediated immune reactions are progressively impaired and our results confirm published data on changes in aged NZB mice: (a) lowering of NK cell activity, (b) decreased T-cell reactions essentially of helper function (mitogen lymphoproliferative response—data not shown), IL-2 production (Wolos & Smith, 1982), generation of CTL (Falkoff, Scanulli & Dutton, 1978); (c) a decreased amount of TSF (Bach *et al.*, 1973); (d) a reduced percentage of Thy-1^+ cells (Hirano & Nordin, 1976); (e) an increase in the number of mice that have abnormal levels of anti-DNA in their serum (Lambert & Dixon, 1970). In addition, we demonstrated an important defect of macrophages in IL-1 production and in their cytostatic capacity (data not shown). To our knowledge, this finding has not been reported before.

The present results extend previous observations that ND has a favourable effect on autoimmune disease (Verheul *et al.*, 1981) and favour the rationale of its action on cell derived from the T lineage: activity of NK cells, which are considered to be in part of prethymic origin, production of IL-2 by T helper cells, T-cell cytotoxicity and TSF release by thymic cells are enhanced or even reconstituted by ND treatment. Moreover, a significant augmentation in the percentage of the Lyt-2^+ T cells (cytotoxic/suppressor subpopulations) occurs. Beside we found that ND strongly stimulates macrophages to release IL-1; a signal which is essential for T cell activation.

On the basis of our findings we postulate that this steroid acts on T-cell differentiation either by exerting a direct effect on the T-cell population or via its effect on another cell population. The effect

of androgen on cell-mediated reactions and on autoimmune phenomena could be mediated through the stimulation of the thymus to release thymic hormones which in turn would induce a stepwise process of maturation of thymocytes and T cells. The presence of receptors for androgens on thymus (Raveché *et al.*, 1980) cells and more precisely on epithelial cells recently reported by Barr *et al.* (1982), and our observation of an increased TSF activity in the serum of ND-treated animals support our hypothesis.

Since functional Ia^+ accessory cells are necessary for proliferation and differentiation of the T cell system, the restoration of IL-1 production by macrophages suggests another possible mechanism of action of ND on the immune system through macrophage regulatory activity. We also found an improved cytostatic activity of macrophages for tumour cells *in vitro* (data not shown). Our data appear to be in agreement with results reported by others (Shear *et al.*, 1981) which show that, in NZB/NZW mice, sex hormones stimulate the macrophages to clear complement-sensitized erythrocytes and polyvinyl pyrollidone and can therefore help in the removal of immune complexes responsible for autoimmune deposits in organs. Thus, the improvement of these functions upon ND administration may enhance the therapeutic effect of the compound.

The reduced capacity of ND-treated animals to develop anti-DNA serum antibodies may also be due to the regulatory activity of an increased number of Lyt-2^+ T suppressor cells.

In conclusion, our observations confirm the importance of both T-cell mediated regulation and of macrophage function in the etiology of autoimmune disease in NZB mice; ND seems to exert its favourable effects at both levels.

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