*Clin. exp. Immunol.* (1980) **41**, 63–72.

# Age- and sex-dependent thymic abnormalities in NZB × SJL $F_1$ hybrid mice

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*(Accepted for publication 16 January 1980)*

## SUMMARY

The cellular organization of the thymus was investigated in 3- and 12-month-old NZB × SJL $F_1$ hybrid (NS) mice. Age-dependent alterations were demonstrated which differed strikingly according to the sex of the animals. In female mice, marked abnormalities of the thymus developed during ageing. They consisted of a more or less pronounced hypertrophy accompanied by histological changes and modifications in the nature of the lymphocyte populations. Three types of qualitative changes were found at 12 months of age: (1) depletion of cortical thymocytes as evidenced by histology, by the evaluation of peanut-agglutinin (PNA) binding and by cell electrophoresis; (2) hyperplasia of the medullary lymphoid tissue, probably reflecting the expansion of a population of mature T lymphocytes. This was further suggested by a rise (up to 60%) in the frequency of lymphocytes lacking both PNA receptor and B cell markers, by an increased proportion (57%) of high electrophoretic mobility (EPM) lymphocytes and by an augmentation of *in vitro* reactivities to phytohaemagglutinin (PHA) and, although to a lesser extent, to concanavalin A (Con A). (3) The appearance of significant numbers of B lymphocytes (up to 20%) as assessed by surface immunoglobulin (sIg) and complement receptor (CR) detection which was accompanied by a vigorous responsiveness of thymus cells to lipopolysaccharide (LPS). None of these abnormalities was seen in the male mice. Instead, the thymus of NS males displayed a nearly normal age-related involution without major change in the proportions of its lymphocyte subpopulations. NS mice thus provide an interesting model of thymic disease influenced by sex-linked factors.

## INTRODUCTION

The normal cellular organization of the thymus reflects its major function which is to provide the inductive stimuli for stem cells to differentiate into immunocompetent T cells (Miller, 1962). It has long been recognized that under pathological situations including leukaemia and autoimmunity this organization may become markedly altered (Metcalf, 1966). The investigation of these thymic alterations appears of particular interest since it might not only allow a deeper understanding of their significance but also provide more insight into the pathways of intrathymic lymphocyte development (Shortman, 1977).

Among such pathological alterations are the occurrence of germinal centres in the medulla (Burnet & Holmes, 1962) and the early thymic atrophy (De Vries & Hijmans, 1967) which occur in NZB mice, a strain known to produce spontaneously a variety of autoantibodies (Talal & Steinberg, 1974). Changes in the cellular organization of the thymus involving a progressive infiltration by

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0099-9104/80/0700-0063$02.00 

populations of mature B and T lymphocytes have also been shown to take place during ageing in SJL mice (Ben-Yaakov & Haran-Ghera, 1975; Dumont, 1978b). However, in both cases, the observation and interpretation of the thymic lesions are hampered by a high rate of early mortality due to the development of either haemolytic anaemia in NZB mice or reticulum cell sarcoma in SJL mice (Murphy, 1969). In previous studies, we found that the progeny of the cross between these two strains, namely the NZB × SJL $F_1$ (NS) hybrids, exhibits a high incidence of thymic alterations without dying prematurely (Dumont, 1978b; Dumont & Monier, 1978).

The present work was undertaken as an extension to these preliminary observations. We investigated in more detail the cellular organization of the thymus of NS mice as a function of age and sex. This was achieved by two complementary approaches. Firstly, we studied the histological structure of the organ; secondly, we evaluated various surface and mitogenic markers to identify the intrathymic lymphocyte populations. The results corroborate our previous findings and further demonstrate a striking influence of sex on the appearance of thymic abnormalities.

## MATERIALS AND METHODS

*Mice.* (NZB♀ × SJL/J♂) $F_1$ hybrids (NS) were raised in our laboratory from inbred parental strains. Female NZB mice were obtained directly from the Centre d'Elevage des Animaux de Laboratoire, CNRS, Orléans-la-Source, France, and male SJL/J mice from our own colony originating from the Jackson Laboratory, Bar Harbor, Maine. All animals were housed under conventional conditions.

*Thymus dissection and histology.* Mice were killed by ether anaesthesia. The two thymus lobes were removed, washed in culture medium and dissected with extreme care to remove any adherent lymph nodes. Pieces of tissue for histological examination were fixed in Bouin's fluid and processed and embedded in paraffin wax by conventional methods. Sections were cut at 5 $\mu$m and stained with haematoxylin and eosin.

*Preparation of cell suspensions.* The thymus lobes were gently disrupted in cold RPMI 1640 medium supplemented with 5% heat-inactivated foetal calf serum (RS). Dissociated cells were freed of larger fragments by filtration through a two-layer gauze. Cells were enumerated in a standard haemocytometer to determine the thymus cellularity.

*Detection of lymphocytes bearing surface receptors for peanut agglutinin (PNA).* A fluorescence microscopy technique was used as described earlier (Dumont & Nardelli, 1979). PNA labelled with fluorescein (FITC-PNA) was obtained from Industrie Biologique Française, Clichy. It was dissolved in phosphate-buffered saline (pH 8·0) at a concentration of 100 $\mu$g/ml and stored at −20°C in small aliquots until use. Mixture of $2–5 \times 10^5$ washed thymus cells suspended in 20 $\mu$l RS was performed with 40 $\mu$l FITC-PNA solution followed by incubation for 25 min at 4°C. After two washings, the cell pellet was carefully resuspended in 100 $\mu$l RS and one drop of cell suspension was mounted under a coverslip. Preparations were examined under incident u.v. light in a Zeiss microscope. A minimum of 200 cells were scored in each sample. Cells exhibiting membrane fluorescence in a cap or diffuse pattern were recorded as $PNA^+$ cells.

*Detection of lymphocytes bearing surface immunoglobulin determinants (sIg).* This was done by direct immunofluorescence (Raff, Sternberg & Taylor, 1970) using a FITC-conjugated goat anti-mouse 7S immunoglobulin antiserum (Hyland; Costa Mesa, California). It was confirmed that this reagent stained 85–90% of electrophoretically separated CBA/J splenic B cells and did not react with T cells. Incubation of $2–5 \times 10^5$ washed thymus cells was performed with 1:4 diluted anti-Ig serum for 25 min at 4°C. The cells were washed twice and mounted under a coverslip for examination in incident u.v. light. At least 200 cells were scored in each preparation to establish the frequency of $sIg^+$ lymphocytes.

*Detection of complement receptor (CR) bearing lymphocytes.* A rosette assay was employed. Indicator sheep red blood cells (SRBC) sensitized with anti-SRBC antibodies and complement (EAC) were prepared as described by Bianco, Patrick & Nussenzweig (1970). SRBC and anti-SRBC serum were purchased from the Pasteur Institute, Paris. Fresh CBA mouse serum was used as a source of complement. Lymphocytes and EAC were mixed and incubated for 30 min at 37°C on

a rotator. A positive EAC rosette was defined as a lymphocyte surrounded by three or more SRBC.

*Cell electrophoresis.* Investigation ofthe electrokinetic behaviour of thymus cell suspensions was performed as previously described (Dumont, 1978a, b) by means of a Hannig's type free-flow electrophoresis apparatus (Vap 5; Bender Hobein; Münich, Germany). The number of cells contained in the various electrophoretic fractions was determined in a ZBlc Coulter counter.

*Lymphocyte cultures.* Cultures for assessment of the mitogenic reactivities of thymus cells were set up in flat-bottomed microplates (Falcon 3040). Each well received $10^6$ cells suspended in a volume of 0·2 ml RS. Various doses of concanavalin A (Con A; Calbiochem), phytohaemagglutinin (PHA-P; DIFCO), or lipopolysaccharide (LPS from *E. coli* 0·55 : B·5; DIFCO) were added in triplicate. The plates were incubated at 37°C in a humidified atmosphere of 95% air, 5% $CO_2$. After 24 hr, the cultures were pulsed with 1 $\mu$Ci $^3$H-thymidine ($^3$H-TdR; 27 Ci/mmol, CEA) per well for a further 24-hr period. The samples were collected and washed by means of a multiple harvester MASH II (Microbiological Associates; Bethesda). The dried filter discs were eluted with 4 ml of scintillation fluid and counted in an SL30 Intertechnique scintillation spectrometer. The results of radioactivity measurements were expressed as mean c.p.m. of triplicates. Statistical analysis of the data was done using Student's *t*-test.

## RESULTS

*Size and cellularity of the thymus*

Typical thymus glands from male and female NS hybrids at 3 and 12 months of age are shown in Fig. 1. In old males, the thymus was consistently smaller than in young animals. In marked contrast, the thymus was often larger in females at 12 than at 3 months. Moreover, in about 30% of the old female mice, a prominent asymmetry of the thymus could be noted, one lobe being more hypertrophied than the other (Fig. 1d).

The numbers of lymphocytes which were recovered after dissociation of the thymus in a series of 46 to 87 individual mice are plotted in Fig. 2. It can be seen that in the males, the mean thymus cellularity sharply decreased at the age of 12 months. Instead, in the 12-month-old females, although exhibiting a wide dispersion the various cellularity values averaged a level close to that recorded at 3 months.

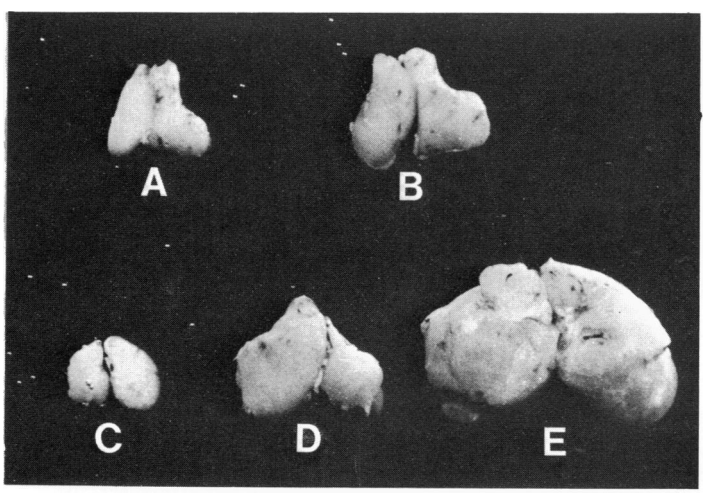


**Fig. 1.** Macroscopical appearance of the thymus from NS hybrid mice; (A) 3-month-old male, (B) 3-month-old female, (C) 12-month-old male, (D and E) 12-month-old females. Note the asymmetric development of the two thymic lobes in (D).

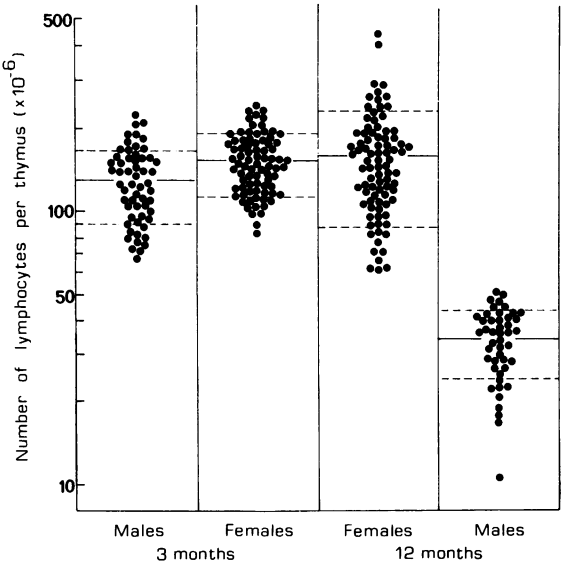


**Fig. 2.** Cellularity of the thymus in male and female NS mice at the ages of 3 and 12 months. Each point represents the number of lymphocytes yielded by the thymus from a single mouse. Horizontal bars represent mean (——) ± standard deviation (- - -).

*Histology of the thymus*

As depicted in Fig. 3, at the age of 3 months the thymus from both male and female NS mice show normal cortico-medullar structure. Despite a shrinkage of the organ, this overall structure was strikingly preserved in the 12-month-old males. The situation was quite different for the 12-month-old females. There, the cortex was severely depleted or even completely absent while the medulla displayed large accumulations of lymphoid cells with a reduction in the numbers of epithelial cells. Lymphoid follicles were frequently conspicuous in this hyperplasic medullary region. Numerous vessels filled with densely stained small lymphocytes were also present.

*Surface characteristics of the thymus cells*

Various surface markers allowing the identification of lymphocyte subpopulations were examined in lymphoid cell suspensions prepared from the thymus of young and old NS mice.

High frequencies (76–77%) of $PNA^+$ cells, which are known to represent the steroid-sensitive cortical thymocytes (Reisner, Linker-Israeli & Sharon, 1976; London, Berrih & Bach, 1978) were encountered in the thymus of young NS mice of both sexes (Table 1). Such frequencies were of the same order of magnitude as those we previously observed in other mouse strains (Dumont & Nardelli, 1979). The proportion of $PNA^+$ cells was also elevated (74%) in the thymus of old NS males. In contrast, it was found diminished by more than three-fold in the thymus of the female mice at the age of 12 months.

Less than 1% of the thymus cells from young mice or from 12-month-old males expressed readily detectable sIg or membrane-bound CR, both of which are properties of B cells (Bianco *et al.*, 1970; Unanue *et al.*, 1971). However, mean frequencies as high as 19·5% $sIg^+$ cells and 10·9% $CR^+$ cells were observed in the thymus ce'l suspensions from 12-month-old females. This suggests the occurrence of substantial numbers of B lymphocytes in the thymus of these mice. Furthermore, assuming that the $sIg^+$ cell compartment normally includes most $CR^+$ cells (Scher, Ahmed & Sharrow, 1977) and does not significantly overlap with the pool of $PNA^+$ cells (Roelants *et al.*, 1979), it might be inferred that on average up to 60% of the lymphoid cells contained in the thymus of 12-month-old NS female mice are $sIg^-$, $CR^-$ and $PNA^-$.

Another parameter which permits the distinction of lymphocyte subclasses is the cell surface charge measurable as cell electrophoretic mobility (EPM) (Wioland, Sabolovic & Burg, 1972).

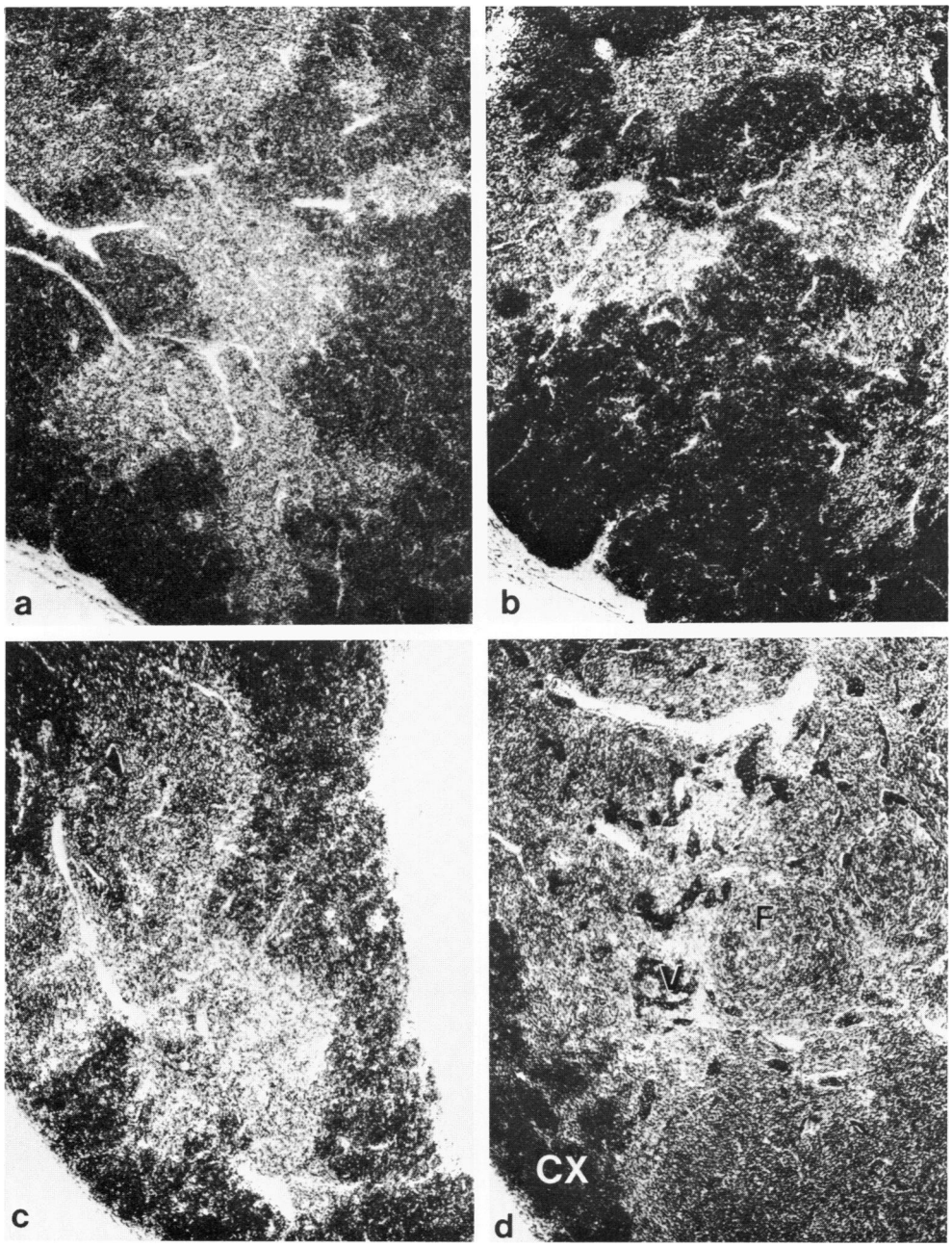


**Fig. 3.** Sections of the thymus from NS hybrid mice. (a) Three-month-old male, (b) 3-month-old female, (c) 12-month-old male. A normal cortico-medulllar structure is evident in all three cases. (d) Twelve-month-old female. Note the atrophy of the cortex (CX) and the presence of a lymphoid follicle (F) and of vessels packed with small lymphocytes (V) in the hypertrophied medulla. (H & E, original magnification × 25.)

**Table 1.** Surface-marker characteristics of thymus cells from 3- and 12-month-old male and female NS mice

| Mice | | | Percentages of thymus cell subpopulations (mean ± s.e.m.) | | |
|---|---|---|---|---|---|
| Age (months) | Sex | Number of experiments* | $PNA^+$ cells† | $sIg^+$ cells† | $CR^+$ cells‡ |
| 3 | Male | 17 | 77·0 ± 1·1 | 0·0 ± 0·0 | 0·2 ± 0·1 |
| 3 | Female | 19 | 75·6 ± 1·6 | 0·6 ± 0·2 | 0·4 ± 0·1 |
| 12 | Male | 28 | 74·3 ± 1·5 | 0·5 ± 0·2 | 0·6 ± 0·2 |
| 12 | Female | 42 | 19·3 ± 1·8 | 19·5 ± 2·0 | 10·9 ± 0·9 |

* Each experiment was done with the thymus cells from a single mouse.
† Assessed by fluorescence microscopy.
‡ Evaluated by EAC rosette formation.

Experiments were conducted to determine the electrokinetic behaviour of the thymus cells from NS mice of different ages. As illustrated in Fig. 4, the electrophoretic distribution profiles of the thymocytes from 3-month-old NS mice were quite reminiscent of those previously reported in other strains (Dumont, 1974; Zeiller *et al.*, 1974; Dumont, 1978a). Thus, a single peak was evident with a distinct skew towards the anode, indicating the existence of a minor cell subpopulation with higher EPM. Such a pattern was also obtained with the thymus cells from 12-month-old males. On the contrary, the thymus cell electropherogram was profoundly altered in the 12-month-old females. In

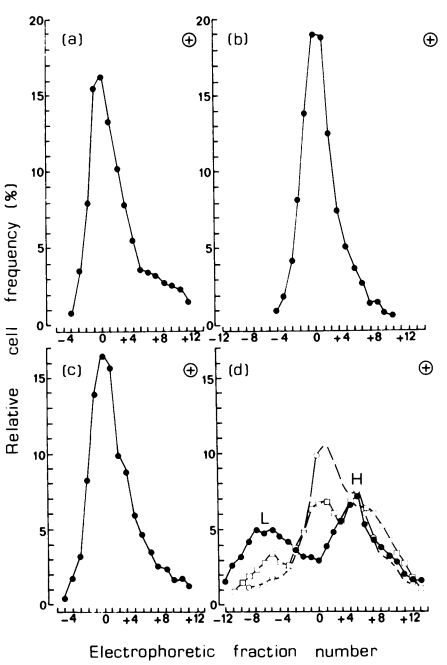


**Fig. 4.** Electropherograms of thymus cells from NS mice. (a) Three-month-old male, (b) 3-month-old female, (c) 12-month-old male, (d) 12-month-old females (three experiments). Each experiment was done with the thymus cells from a single mouse. Fraction number 0 was arbitrarily ascribed to the electrophoretic peak of thymocytes from 3-month-old mice. In (d) note the disappearance of this peak and the appearance of a population with higher EPM (H) and of another one with lower EPM (L).

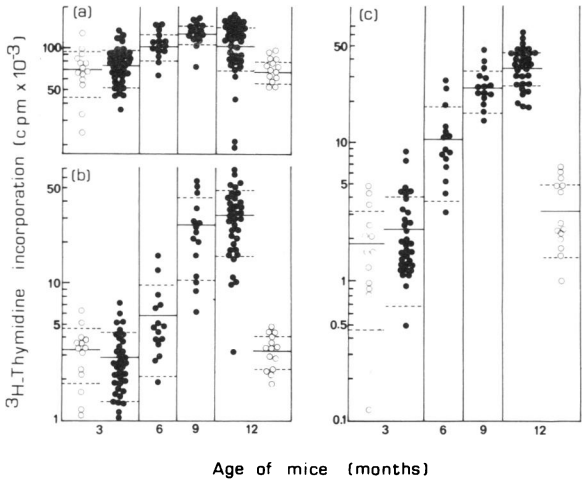


**Fig. 5.** Mitogenic reactivities of thymus cells from male (○) and female (●) NS mice at different ages. (a) Reactivity to Con A (0·5 μg/well), (b) reactivity to PHA (10 μg/well), (c) reactivity to LPS (5 μg/well). The data are expressed as mean c.p.m. of triplicates over background values (cultures without mitogen). Each point represents the results obtained with the thymus cells from a single mouse. Horizontal bars represent mean (——) ± standard deviation (– – –).

most cases, the population of low EPM thymocytes tended to disappear whereas a peak of high EPM cells, presumably corresponding to mature T cells (Zeiller *et al.*, 1974; Dumont, 1978a) was then clearly visible. This latter population was found to account for 57 ± 6% (mean ± s.e.m. of ten experiments) of the aged female thymus cells. Moreover, a new population with an EPM slower than that of typical thymocytes became apparent (Fig. 4d).

*Mitogenic reactivities of the thymus cells*

Thymus cells were cultivated in the presence of three different mitogenic agents tested over a wide range of dilutions. To facilitate comparisons, cells from the different groups of mice were always studied in parallel. The various increments of 3H-TdR uptake induced by optimal doses of mitogens are plotted in Fig. 5. These data are derived from a series of thirty-three experiments.

In all instances, Con A triggered a vigorous enhancement of $^3$H-TdR incorporation by thymus cells. This reactivity was very similar for thymus cells from young male and female and from old male mice. It was significantly augmented ($P < 0{\cdot}001$) with increasing age of the female donors, although in two out of the thirty-five 12-month-old females studied this reactivity was lower than in the young animals.

Thymus cells from 3-month-old NS mice responded poorly to PHA. This finding is consistent with what has been generally observed in normal mice (Janossy & Greaves, 1971; Dumont & Robert, 1976). During ageing, PHA reactivity remained low in the males whereas it dramatically rose in the females, reaching a highly significantly increased mean value at 12 months ($P < 0{\cdot}01$). Thus, in thirty-four out of the thirty-five old female mice examined, the stimulation of thymus cell $^3$H-TdR uptake by PHA was 10–15-fold greater than in young mice.

As would be expected from earlier studies (Andersson, Möller and Sjöberg, 1972; Ozato, Adler & Ebert, 1975), the extent of thymus cell responsiveness to the B cell mitogen LPS was very limited in the 3-month-old mice of both sexes. In the course of ageing, this LPS reactivity increased slightly in the male NS mice ($P < 0{\cdot}05$) but much more strongly in the female NS mice ($P < 0{\cdot}001$). Thus, at 12 months of age, proliferative responses to LPS which were by now up to 20-fold higher than at 3 months of age, were repeatedly recorded with thymus cells from female donors.

## DISCUSSION

The results reported here confirm and extend our previous observations (Dumont, 1978b; Dumont

& Monier, 1978) that in female NS mice profound modifications affect the cellular organization of the thymus in the course of ageing.

One of these changes concerned a severe atrophy of the cortical tissue. Concomitantly, a drop in the frequency of cells carrying surface receptor for PNA was noted. This agrees well with the idea that $PNA^+$ thymocytes represent mainly cortical lymphocytes (Reisner *et al.*, 1976; London *et al.*, 1978). Cell electrophoresis data further strengthened the notion that cortical thymocytes, characterized by a relatively low EPM (Zeiller *et al.* 1974; Dumont, 1978a), tended to disappear in the aged NS females.

A second type of alteration was histologically detectable as a marked hyperplasia of the medullary lymphoid tissue. Indeed, this hyperplasia accounted for the maintenance and eventual augmentation of the size and cellularity of the thymus at 12 months of age. The assessment of surface markers indicated that, at this time, many of the thymus cells (60%) were devoid of both PHA receptor and B cell markers. This observation, taken together with the fact that the proportion of high EPM cells was increased, would suggest that a majority of the cells located in the hypertrophic medulla were mature T cells. In support of this view, strong mitogenic responsiveness to PHA was regularly found with thymus cells from aged female mice. Since concurrently the reactivity to Con A was less intensely enhanced or even diminished, it seems likely that the immunocompetent T cells which expanded in the thymus of old females belonged to a low Con A-responsive subset (Stobo, 1972).

Another remarkable feature of the old female thymus was the frequent occurrence of lymphoid follicles within the medullary area. Such structures usually contain cells of the B lymphocyte lineage (Gutman & Weissman, 1971). Indeed, appreciable frequencies of B lymphocytes (19·6% $sIg^+$ cells and 10·9% $CR^+$ cells) were encountered in the cell suspensions prepared from the thymus of 12-month-old females. Moreover, in a separate study (manuscript in preparation), we demonstrated that the new electrophoretic cell type emerging in the late stages of the thymus evolution and characterized by a lower EPM than normal thymocytes essentially included lymphocytes with B cell properties. Quite consistent with this evidence for the presence of B lymphocytes in the thymus of the aged females was the abnormally high thymus cell reactivity to LPS that could be detected already at 6 months and which still increased upon further ageing.

It therefore appears that while the normal thymocyte population regresses, large numbers of lymphocytes with B and T cell attributes accumulate in the thymus of ageing female mice. The significance of such alterations remains obscure. The B cells which infiltrated the thymus of these mice very probably originated from the periphery. However in the case of T cells it is not clear whether they were generated *in situ* from intrathymic precursors or represented migrants from other lymphoid organs.

In experiments to be published (Monier & Dumont, manuscript in preparation), we have demonstrated that NS female mice are prone to develop spontaneous autoimmunization as manifested by the formation of anti-nuclear and anti-DNA antibodies and by the deposition of immune complexes in the skin and kidneys. In such a context, an attractive hypothesis would be to consider the NS female thymic lesions as the expression of an autoimmune reaction directed towards some antigenic component of the thymus. Thus, B and T cells with specificity against this constituent might be attracted by the thymic stroma which they would actively infiltrate. Another possibility might be that the mechanism which normally prevents peripheral lymphocytes from entering the thymus (Gowans & Knight, 1964; De Sousa, 1978) becomes altered in such a way that B and T cells are non-specifically entrapped in the organ. Thymus graft experiments as well as the study of the ecotaxis of lymphocytes (De Sousa, 1978) isolated from the thymus and other lymphoid tissues of aged NS female mice could be used to validate these hypotheses.

At any rate, all of the alterations mentioned above were undetectable in the thymus of male NS mice, at least until the age of 12 months. In these animals, the thymus followed an age-related involution sequence comparable to that described in normal mouse strains (Metcalf, 1966; Yunis, Fernandes & Good, 1978) and despite a pronounced quantitative reduction, its lymphocyte content remained qualitatively almost unchanged.

The development of thymic abnormalities in NS mice is therefore clearly influenced by sex-linked factors. These factors might be of two types: either purely genetic, i.e. associated with genes

of the X or Y chromosomes which would control directly the behaviour of the thymus, or of a hormonal nature. The fact that in unpublished observations we found the same kinds of thymic alterations in the reverse SJL♀ × NZB♂ $F_1$ hybrids, with the same female predominance as in the NS hybrids, renders the first alternative unlikely. It seems more probable that these sex-related differences in thymus evolution reflect the action of sex hormones. Several studies have shown that the lupus-like syndrome of NZB × NZW $F_1$ mice develops earlier and with greater intensity in the females than in the males (Melez, Reeves & Steinberg, 1978; Roubinian *et al.*, 1978). In this case, an accelerating effect of androgens and a suppressing effect of oestrogens could both be invoked to explain the sex differences in the expression of autoimmunity. That a similar situation might exist for the thymic abnormalities of NS mice certainly deserves further attention. We are currently investigating such a possibility by means of castration and sex hormone reconstruction experiments.

This work was supported by the INSERM (ATP 71-78-103). We thank Mr Roger Barrois for excellent technical assistance, Mr Michel Claude for breeding NS mice, and Miss Josiane Bara for typing the manuscript. We are also grateful to Drs J. C. Monier, R. M. Parrache and J. Duheille for helpful discussions.

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