Clin. exp. Immunol. (1975) **20,** 323–338.

ROLE OF THE THYMUS IN PROGRAMMING OF NEUROENDOCRINE FUNCTIONS

W. PIERPAOLI AND H. O. BESEDOVSKY*

Schweizerisches Forschungsinstitut, Medizinische Abteilung, Davos-Platz, Switzerland

(*Received* 12 *August* 1974)

SUMMARY

Specific derangements of thyroid and gonadal functions were observed in athymic nude and neonatally thymectomized mice. Such endocrine alterations are already established during the perinatal period and maintained through adult life. Passive transfer of lymphoid cells from normal donors does not prevent such alterations. In nude mice thymus implantation at birth fully reconstitutes oestrogenic function, but thyroid and progestational functions remain defective. Peripheral endocrine glands (thyroid, adrenals and ovaries) respond normally to ACTH, TSH and LH. Thus the thymus may well have a basic role in the organization of the adult hypothalamus–pituitary axis for thyroid and sexual functions.

INTRODUCTION

After a considerable hiatus there is now fairly general agreement that the thymus secretes hormonal factors which affect the development of immune capacity (Davies & Carter, 1973). On the other hand, it is firmly established that practically all hormones participate in the homoeostatic control of most cell functions by synergistic or antagonistic interaction. It therefore seems reasonable that the hormones attributed to the thymus would also influence synthesis, secretion, blood levels or metabolism of other hormones.

It is known that the presence of the thymus during early stages of ontogenic development in mammals influences profoundly the immune capacity, but it is less generally appreciated that the thymus also modulates certain endocrine functions which in turn influence immunological maturation. This correlation has been amply discussed in our previous work (Pierpaoli & Sorkin, 1967; Pierpaoli, Fabris & Sorkin, 1970; Bianchi, Pierpaoli & Sorkin, 1971; Pierpaoli & Sorkin, 1972a); recent findings in athymic nude mice also confirm the postulated endocrine action of the thymus on other endocrine functions in early ontogeny (Pierpaoli & Sorkin, 1972b; Besedovsky & Sorkin, 1974).

* Present address: Universidad Nacional de Rosario, Facultad de Ciencias Medicas, Santa Fe 3100, Rosario, Argentina.

Correspondence: Dr Walter Pierpaoli, Schweizerisches Forschungsinstitut, Medizinische Abteilung, 7270 Davos-Platz, Switzerland.

Removal of the thymus in mice any time subsequent to 48–72 hr after birth does not result in any apparent immediate alteration of either endocrine or immune functions. It is therefore likely that the most critical endocrine function of the thymus is exerted in a very circumscribed period between the last days of pregnancy and the very first days of life in mice and rats, when critically important steps in the development and programming of certain neuroendocrine functions of the brain are taking place (Young, 1961; Harris, 1964, Eayrs, 1966; Levine & Treiman, 1969; Jost, 1969). It is also at this particular phase of development that receptors for hormones are sequentially appearing in the central nervous system. This definitive organization of certain hypothalamic endocrine functions establishes the final hormonal pattern of the individual, sex included (Bassett, Thorburn & Wallace, 1970; Alexander *et al.*, 1971; Gomez, 1971; Brown-Grant & Sherwood, 1971; Jost *et al.*, 1973).

Evidence favouring this endocrine organs–thymus interdependence during early ontogeny is provided by the established findings that in those species in which neonatal thymectomy does not produce any evident impairment of the immune capacity, e.g. guinea-pig, sheep, rabbit and man (Solomon, 1971), neuroendocrine functions are already fully developed at birth (Jost, 1969). In contrast, these functions are not yet organized in newborn mice, hamsters and rats (Jost, 1969), three species in which neonatal thymectomy produces in parallel striking alterations in immune competence (Solomon, 1971). Accordingly the mouse, and especially the athymic nude mouse and the neonatally thymectomized mouse, constitute distinctive and appropriate models for study of a postulated ontogenetic correlation between the thymic hormones, the development of endocrine functions and the proposed link between these early endocrine thymic functions and the development of the immune capacity.

To verify this hypothesis, experiments were devised exploring the hormonal conditions during perinatal age of athymic and neonatally thymectomized animals and the influence of passive transfer of lymphoid cells and of thymus implantation on endocrine parameters of thymus-deprived animals.

MATERIALS AND METHODS

Animals

Homozygous, hairless, athymic mice (BALB/c, nu/nu) and their heterozygous normal-haired littermates (nu/+), originally obtained as pathogen-free animals from Gl. Bomholtgård, Laboratory Animals and Research Centre, Ry, Denmark and maintained in conventional conditions in our animal house as a closed colony were used. These mice are known to lack the capacity to respond to certain antigens with normal antibody production and do not reject allogeneic or heterogeneic grafts (Rygaard, 1973). They were kept in air-conditioned quarters at a temperature of 25–26°C, given pellets and water *ad libitum*. Newborn, athymic nude mice were separated from haired littermates in order to eliminate competition for milk and thus improve their nutritional status. For some determinations of thyroxine, sera obtained from neonatally thymectomized, sham-operated, germ-free NMRI mice from the animal farm of Ciba-Geigy AG, Sisseln, Switzerland were used. Also conventional outbred Charles River mice were used for hormone determinations in neonatally thymectomized mice.

Growth curves

To evaluate growth pattern of nude and haired BALB/c mice, four litters were reduced to eight mice each. Two of these litters were composed of four nude and four haired males and two litters of four nude and four haired females. Four other groups of nude and haired mice similarly composed were injected on day 1 intraperitoneally (i.p.) with 20×10^6 spleen cells from young adult normal haired male and female BALB/c mice. For evaluation of the effect of thymus implantation on the growth pattern of nude mice, two litters were composed of eight male or female nude mice of which four were thymus-implanted at birth and four sham-operated.

Thymectomy

Neonatal thymectomy was performed by the usual suction technique within the first 12 hr of life. Animals were anaesthesized by cooling them in ice.

Skin grafts

Full-thickness skin grafts from inbred C57BL/6 mice were transplanted on the back of nude mice. The graft was fixed with eight silk sutures, covered with sterile Vaseline-impregnated gauze and a light plastic corset was applied. The corset was opened after 7 days and the viability of the graft was followed by daily inspection.

Thymus implantations

Male and female newborn nude mice were implanted subcutaneously (s.c.) into the armpit with one or two intact thymuses from male or female normal littermates. The wound was sutured with silk and sprayed with plastic protective film. Inspection assured that the stitches were not removed by the mother and that the transplanted thymuses were retained.

Inoculation of cells

Suspensions of cells were prepared from spleens of normal young adult BALB/c mice. Spleens were teased in tubes with loose-fitting Teflon pestle using as medium cooled saline phosphate-buffered solution (PBS), pH 7·4. Cells were filtered through gauze and washed four to five times with cold PBS. Tests with trypan blue showed that over 95% of the cells were viable. Quantities of 20×10^6 cells in 0·1 ml PBS were injected into newborn nude or normal mice. To prevent leakage, the needle was inserted s.c. at the jugulum and the cells were released i.p. under the liver.

Determinations of hormones

Partial or final bleedings were performed under strictly standardized conditions. The animals were always bled between 10.00 a.m. and noon. They were rapidly anaesthesized with ether, blood was taken from the retro-orbital plexus with a Pasteur pipette and pools prepared from the blood of four or more animals. Unless otherwise indicated, blood from male or female animals was kept separate. When serum was collected from 6- or 14-day-old mice, blood was obtained from the jugular vein of ether-anaesthesized animals. For progesterone determinations, plasma was obtained by collecting the blood in heparinized tubes. The blood was allowed to clot at room temperature for 2 hr, centrifuged at $+5°C$

and sera frozen and stored at −20°C. Thyroxine, corticosterone, testosterone, progesterone and 17-β-oestradiol were determined by well-known and standardized techniques* (Murphy & Jachan, 1965; Murphy, Pattee & Gold, 1966; Kliman & Peterson, 1960; Mueller, 1965; Buus, 1968; Anderson, 1970; Uettwiller, 1970; Demetrious & Austin, 1971; Mikhail *et al.*, 1970; Abraham, 1969, 1974). In some cases determinations were performed on sera of individual animals. The data express the average values of two or more determinations.

Injection of pituitary hormones

Thyrotropin (NIH-TSH-B6, bovine; 2·54 USP units/mg) and luteinizing hormone (NIH-LH-S18, ovine; 1·03 NIH-LH-S1 units/mg) were generously provided by the National Institute of Arthritis and Metabolic Disease, Pituitary Hormone Distribution Program, Bethesda, Maryland. Groups of three 40-day-old male nude mice and normal haired males were injected i.p. with 200 μg of TSH. Control nude and haired mice were injected with 200 μg of bovine serum albumin (BSA). Blood was taken 1 hr after the injection. The same mice were injected again 8 hr later with the same amounts of TSH or BSA, food was withheld for 14 hr and the animals exsanguinated 22 hr after the first injection. Sera were used for determination of thyroxine (T-4). Groups of three 60-day-old male athymic nude or normal haired mice were injected i.p. with 200 μg of TSH or BSA, fasted for 15 hr, injected again with the same amount of TSH or BSA and exsanguinated 1 hr later for determination of thyroxine and lipid levels.

Groups of three 50-day-old female nude or haired mice were injected i.p. with 200 μg of LH and blood was taken 1 hr later. The same mice were then injected daily with 200 μg of LH for 7 days and then exsanguinated. Nude or haired controls of the same age and sex were analogously treated with the same quantities of ovine serum albumin (OSA). Plasma was obtained for determination of progesterone.

Histology

Full-thickness skin was removed from the back of 14-day-old nude and haired mice, fixed in Bouin's fluid, embedded in paraffin. Sections were stained with haematoxylin and eosin and examined.

Adrenal function in vitro

Groups of twelve, 3-month-old male athymic nude mice and normal haired mice of the same age and sex were used. The mice were killed by cervical dislocation, the adrenals were isolated, freed from the fatty and connective tissue under a dissection microscope, each gland was cut into two pieces and the tissue immersed in cooled Ringer–bicarbonate–glucose solution, pH 7·3. The tissue was maintained in the cold Ringer until the initiation

* In view of the special problems peculiar to hormone determinations, including variability and evaluation of the results, collaboration was established with highly specialized endocrinological laboratories. This has permitted internal comparisons and also objective criteria to judge reliability of data on the basis of identical coded samples sent simultaneously to two or even three different laboratories and to repeat determinations in some instances. We are indebted to Dr Max Keller of Hoffman-La Roche & Company, AG, Diagnostica, Schweizerhalle for determinations of corticosterone, testosterone, progesterone, 17-β-oestradiol and thyroxine; to Dr Jürg Müller, Steroidlabor, University of Zurich, for some determinations of corticosteriods; to Dr H. Kohler and Mr R. Kretschmer, Central Chemical Laboratory, Inselspital, Bern, for determinations of thyroxine and lipids; and to Dr Paul Keller, Hormone Laboratory, Frauenklinik, University of Zurich for determinations of progesterone and 17-β-oestradiol.

of the incubation time. The final volume of the incubation fluid was 3·1 ml. The glands were incubated at 37·5°C for 10 min with shaking and flow or air with 5% CO_2; the flasks were then closed and the incubation was continued for 110 min in the shaking bath. The reaction was terminated by cooling the flasks with ice, the supernatant was filtered through gauze and the wet weight of the glands was determined. Every flask contained twelve half adrenals from either athymic or normal haired mice. One i.u. of ACTH (Synacthen, Ciba-Geigy AG, Basel) was added to some of the flasks. Corticosteroids present in the incubation fluid were determined. Values of corticosteroids are expressed as micrograms of steroid/100 mg of adrenal tissue/2 hr incubation.

RESULTS

Neonatal growth curves

As is evident from Fig. 1, athymic nude mice raised under conventional conditions together with their normal haired littermates, display a retarded growth rate during the first few days of life. The same neonatal retardation of growth has been observed in specific pathogen-free and germ-free nude mice when raised in litters containing haired littermates (personal observations). Isolation at birth of athymic newborn nude mice from their haired normal littermates eliminates competition for milk and improves their nutritional state, almost normalizing their growth. Thymus implantation at birth in nude mice (litters composed of nude mice only) does *not* significantly improve their growth pattern in the first 2 weeks of life (Fig. 1). Intraperitoneal inoculation of 20×10^6 spleen cells from normal haired adult BALB/c mice into newborn nude mice raised together with their haired littermates does not influence their growth rate in the first 2 weeks of life (Fig. 1).

Levels of hormones in blood of athymic mice

Table 1 summarizes the data on the remarkable reduction in serum thyroxine in 6-day-old nude mice as compared to their normal haired littermates. Eight days later the level of thyroxine is still reduced although to a lesser degree, and abnormally low levels of thyroxine are then maintained throughout the whole life span. Testosterone levels are also significantly diminished in male and female 6-day-old nude mice and in 14-day-old male nude mice (Table 2). On the other hand, levels of testosterone in adult nude mice show small variations as compared to normal mice of same age and sex. Corticosterone levels in blood of 6-day-old nude mice do not differ significantly from those in normal mice, while at 14 days of age, athymic mice have a level of corticosterone which is more than doubled in males compared with normal mice of the same age and sex (Table 3). A remarkable reduction in the amount of this steroid is observed in ageing nude mice (8 months old). Levels of progesterone are normal in prepubertal female nude mice and profoundly reduced in sexually mature athymic female mice (Table 4). Levels of 17-β-oestradiol in post-pubertal age in nude mice are much lower than in normal haired females (Table 5).

Levels of hormones in neonatally thymectomized mice

Neonatal thymectomy in newborn mice mimics the hormonal conditions of nude mice. Levels of thyroxine are lower in blood of conventional or germ-free neonatally thymectomized mice than in sham-operated controls (Table 1). The levels of corticosterone, pro-

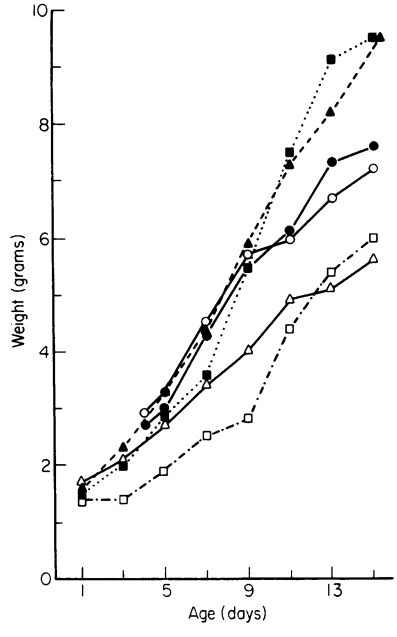

FIG. 1. Postnatal growth curve of athymic and normal haired BALB/c mice. All litters were reduced at birth to eight mice. In the experiments in which mice were untreated or inoculated with spleen cells, litters composed of four athymic and four haired BALB/c mice of the same sex were kept in single cages. The total number of nude or haired untreated mice was sixteen (four litters). In the experiments in which spleen cells were transferred, two groups of male and female, nude and haired mice (eight mice in each group, two litters) were inoculated intraperitoneally in the 1st day of life with 20×10^6 spleen cells from normal adult haired BALB/c donors. Two other litters were reduced to eight nude mice each and the haired newborns were used as thymus donors. In each litter, four nude mice were implanted subcutaneously with one intact thymus from a haired littermate and four others were sham-operated. Reconstitution of cell-mediated immunity in athymic mice injected at birth with spleen cells or implanted with thymus was validated by grafting them at 40 days of age with skin from C57Bl/6 donors. Untreated athymic nude mice (△) and their normal haired litter-mates (▲). Athymic nude mice injected at birth intraperitoneally with 20×10^6 spleen cells from adult syngeneic normal haired donors (□) and their normal haired litter-mates injected similarly with the same number of cells (■). Athymic nude mice implanted at birth with thymus (○) and their sham-operated nude litter-mates (●).

TABLE 1. Altered thyroxine levels in athymic and thymectomized mice

Age (days)	Sex	Normal (nu/+)	Athymic (nu/nu)	Sham-operated	Neonatally thymectomized
6	F	9·50	6·14	—	—
6	M	11·30	7·70	—	—
14	F	12·10	10·40	12·00	10·50
14	M	12·80	10·10	12·40	10·30
7–11–12–19	M+F*	—	—	9·70	7·80
26–31	M+F*	—	—	8·90	7·00
30	M	9·60	8·50	—	—
40	M	11·70	8·30†	8·50	6·30
44	M	12·00	8·00	—	—
52	M	8·90	5·20	—	—
70	F	9·00	6·20†	—	—
70–80	F	10·40	5·80	—	—
85–90	F	10·70	6·40	—	—
180–240	M	10·20	7·20	—	—

Thyroxine (T–4) is expressed as micrograms per 100 ml serum.

* NMRI mice, born, thymectomized and maintained in germ-free conditions.

† Transplanted at birth with thymus from normal litter-mates and with normalization of cell-mediated immune reactions.

TABLE 2. Altered testosterone levels in athymic mice

Age (days)	Sex	Normal (nu/+)	Athymic (nu/nu)
6	F	87	37
6	M	320	240
14	F	26	26
14	M	180	80
90	M	180 160*	220
100	M	200	220

Testosterone is expressed as nanograms per 100 ml serum.

* Transplanted at birth with thymus from normal litter-mates and with normalization of cell-mediated immune reactions.

TABLE 3. Altered corticosterone levels in athymic and thymectomized mice

Age (days)	Sex	Normal (nu/+)	Athymic (nu/nu)	Sham-operated	Neonatally thymectomized
6	F	0·92	0·81	—	—
6	M	1·30	1·14	—	—
14	F	1·20	1·55	7·80	8·50
14	M	1·32	2·63	12·40	15·30
42*	M	20·20	18·20	—	—
			22·10†		
56*	M	27·20	39·80	—	—
240*	M	29·20	15·90	—	—

Corticosterone is expressed as μg/100 ml serum.

* Mice aged 42, 56 and 240 days were bled in the afternoon hours.

† Transplanted at birth with thymus from litter-mates and with normalization of cell-mediated immune reactions.

TABLE 4. Altered progesterone levels in athymic and thymectomized mice

Age (days)	Sex	Normal (nu/+)	Athymic (nu/nu)	Sham-operated	Neonatally thymectomized
35*	F	0·45	0·52	—	—
40	F	3·00	0·66	—	—
45–50	F	—	—	1·13	0·24
54	F	0·43	0·14	—	—
			0·17†		
56	F	0·29	0·06	—	—
			0·15†		
56‡	F	0·95	0·26	—	—
65	F	1·62	0·63	—	—

Levels of progesterone are expressed as micrograms per 100 ml plasma.

* Prepubertal age.

† Thymus-implanted at birth.

‡ Specific pathogen-free mice

TABLE 5. Altered 17-β-oestradiol levels in athymic and thymectomized mice

Age (days)	Sex	Normal (nu/+)	Athymic (nu/nu)	Sham-operated	Neonatally thymectomized
35*	F	0·96	1·45	—	—
45–50	F	—	—	2·47	2·18
54	F	2·05	1·08	—	—
			2·18†		
56	F	2·20	1·64	—	—
			2·62†		
56‡	F	2·35	1·42	—	—
65	F	3·34	0·29	—	—

17-β-oestradiol is expressed as nanomoles per litre of serum.

* Prepubertal age.

† Thymus-implanted at birth.

‡ Specific pathogen-free mice.

gesterone and 17-β-oestradiol in perinatal age follow a pattern similar to that of nude mice (Tables 3, 4, 5).

Effects of passive transfer of lymphoid cells or thymus implantation at birth on cell-mediated immunity in adult nude mice

Both procedures completely normalize cell-mediated immune reactivity of nude mice as evidenced by the acquired capacity to reject allogeneic skin grafts within a normal time (8–12 days).

Effects of passive transfer of lymphoid cells or thymus implantation at birth on life span and levels of hormones in blood of adult nude mice

The general nutritional state of nude mice injected at birth with syngeneic lymphoid cells is not significantly improved over that of their untreated nude littermates, nor is their life span prolonged. Levels of thyroxine in serum are not modified by passive transfer of lymphoid cells. Also, fertility of females is not affected and the delay in puberty is not shortened. Therefore the only parameter investigated which is normalized is their cell-mediated immune capacity. Neonatal implantation of thymus, on the contrary, besides normalizing the cell-mediated immune reactions in nude mice, improves their general vigour in young adult life, growth rate, life span and fertility of females (expressed as number of litters per female—three to four litters instead of 0 to 1 under conventional conditions). In fact, while the levels of thyroxine and progesterone are not significantly affected by thymus implantation at birth, this intervention does normalize levels of 17-β-oestradiol (Tables 1, 4 and 5).

Test for adrenal function in vitro

The data given in Table 3 show that the level of corticosterone is slightly decreased in 6-day-old nude mice, rises over the normal values in 14-day-old and adult nude mice and is starkly reduced in ageing nude mice (over 8 months). *In vitro* tests show however that the adrenal cortex of nude mice can respond normally to stimulation with ACTH (Table 6).

TABLE 6. *In vitro* production of corticosteroids by adrenal glands of nude and normal BALB/c mice

Adrenal donors	Corticosterone*	Aldosterone*	Deoxycorticosterone*
Athymic mice	4·65 ± 1·83	1·20 ± 0·35	0·13 ± 0·07
Normal mice	4·25 ± 1·25	1·24 ± 0·16	0·04 ± 0
Athymic mice + ACTH	77·94 ± 11·51	5·22 ± 0·35	4·26 ± 0·59
Normal mice + ACTH	59·83 ± 0·97	4·12 ± 0·04	4·05 ± 0·17

* The values of the steroids are expressed as micrograms of steroid per 100 mg of adrenal tissue per 2 hr of incubation (mean value of two flasks ± standard error).

This indicates that biosynthesis of corticosteroids is normal in the adrenals of nude mice and that variations in blood levels must therefore be attributed to an abnormal hypothalamic regulation or to peripheral interactions or to compensatory mechanisms involving other hormones such as thyroxine or gonadal steroids whose levels are lower in nude mice.

Effect of injection of thyrotropic hormone on levels of thyroxine and lipids

Injection of TSH into athymic and haired mice induces a rapid increase in levels of thyroxine. This increase is particularly pronounced after fasting and a second inoculation of TSH (Tables 7 and 8). Triglycerides, which are very low in fasting nude mice are not affected by TSH injection whereas in normal haired mice TSH reduces their blood triglycerides levels.

TABLE 7. Increase in levels of thyroxine in peripheral blood of athymic and normal BALB/c mice produced by thyrotropic hormone (TSH)

	Age (days)	Sex	Thyroxine*	Thyroxine† after fasting
Nude+BSA	40	M	7·1	5·5
Nude+TSH	40	M	8·1	10·5
Haired+BSA	40	M	9·3	8·0
Haired+TSH	40	M	11·8	15·8

Values of thyroxine (T-4) are expressed as micrograms per 100 ml of serum.

* One hour after injection of 200 μg of BSA or TSH.

† Twenty-two hours after the first injection and 14 hr after a second injection of either 200 μg of BSA or TSH and fasting.

TABLE 8. Effect of thyrotropic hormone (TSH) on levels of thyroxine and lipids in peripheral blood of athymic and normal BALB/c mice

	Age (days)	Sex	Thyroxine	Phosphatides	Cholesterol	Triglycerides	Total lipids
Nude+BSA	60	M	4·7	166	150	28	434
Nude+TSH	60	M	9·1	104	136	39	362
Haired+BSA	60	M	6·9	168	184	385	844
Haired+TSH	60	M	11·5	168	167	150	583

Two hundred micrograms of either BSA or TSH were injected twice, at time 0 and 15 hr later. Mice were killed after 16 hr of fasting and 1 hr after the second injection. Thyroxine (T-4) is expressed as micrograms per 100 ml of serum. Phosphatides, cholesterol, triglycerides and total lipids are expressed as milligrams per 100 ml of serum.

Effect of injection of luteotropic hormone on levels of progesterone

Injection of LH in haired and athymic nude mice leads to a sharp rapid increase in blood levels or progesterone within 1 hour after the inoculation. When the treatment is prolonged for a few days, the levels of progesterone in athymic mice reaches values comparable to those of normal female mice (Table 9).

TABLE 9. Increase in levels of progesterone in peripheral blood of athymic and normal BALB/c mice produced by luteotropic hormone (LH)

	Age (days)	Sex	Progesterone* after 1 hr	Progesterone* after 7 days
Nude+OSA	50 and 57	F	0·17	0·21
Nude+LH	50 and 57	F	0·71	1·36
Haired+OSA	50 and 57	F	0·24	0·60
Haired+LH	50 and 57	F	1·91	1·71

Progesterone is expressed as micrograms per 100 ml plasma.

* Blood was taken 1 hr after injection of 200 μg of LH or OSA and again from the same mice after 7 days treatment with 200 μg of LH or OSA per day.

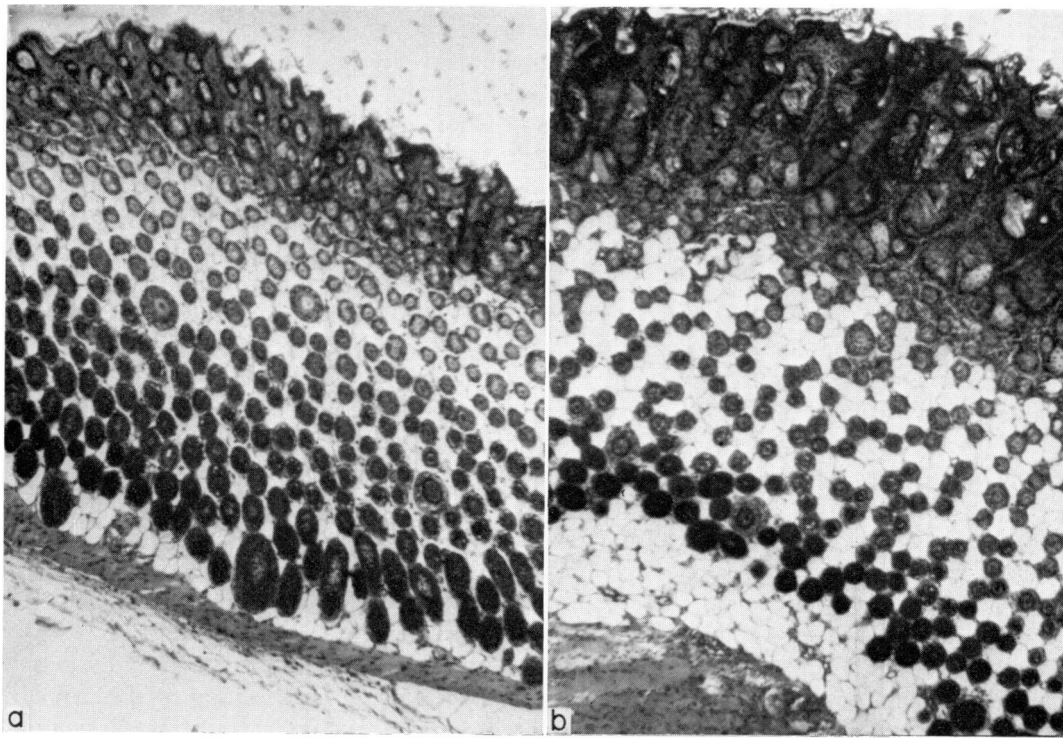

FIG. 2. Skin sections of (a) 14-day-old normal haired and (b) athymic nude BALB/c mouse. Besides the obvious reduction in the number and atresia of *follicula pilifera*, the remarkable thickness and keratinization of the epidermis of athymic nude mice is noteworthy. (Haematoxylin and Eosin; magnification ×9·8.)

Histological findings

The skin of 14-day-old athymic mice shows an obvious reduction in number and atrophy of hair follicles. The skin of nude mice is much thicker than that of haired mice, due to massive enlargement and keratinization of the epidermis (Fig. 2).

DISCUSSION

This investigation on the endocrine status of neonatally thymectomized and athymic nude mice has yielded findings in harmony with our hypothesis that the thymus participates in the functional differentiation of the brain and in the programming of certain endocrine functions. These basic effects are exerted by thymic influence changing the endocrine status during foetal and perinatal life. The secretion of thyroxine and gonadotrophins are particularly affected, and this results in major consequences on general development.

The growth curve in perinatal life can be taken as a comprehensive expression of neuroendocrine and nutritional status, the latter being largely dependent on the former. Impairment of the growth of nude mice raised together with their haired littermates is, in our view, due to their deficient or less mature neuroendocrine capacity. The fact that they grow normally when isolated from their haired littermates is a sign of their inability to compete with normal, more mature, littermates. Full immune capacity induced by passive transfer of immunocompetent lymphocytes to newborn athymic mice does not affect their retarded growth and likewise does not induce changes in blood levels of hormones. This indicates that endocrine and immune functions, although both depend on thymic influence, can be dissociated.

Determinations of hormones in the first days of life confirm a neuroendocrine dysfunction in athymic and neonatally thymectomized mice. Levels of thyroxine and testosterone are abnormally low (Tables 1 and 2). It is now well established that, in mammals, the level of hormones during the perinatal period determines to a large extent the future neuroendocrine activity of the hypothalamus (Young, 1961; Harris, 1964; Eayrs, 1966; Levine & Treiman, 1969; Jost, 1969; Jost *et al.*, 1973; Gomez, 1971; Brown-Grant & Sherwood, 1971). Early in foetal life thyroxine affects the development of the entire brain (Eayrs, 1966; Gomez, 1971). The low blood levels of thyroxine during the perinatal period (6 and 14 days of life) in athymic and neonatally thymectomized mice could therefore adversely influence the animal's future neuroendocrine status. The persisting low levels of thyroxine in adult athymic and neonatally thymectomized mice (Table 1) may thus express an altered hypothalamic function for synthesis and secretion of thyrotropin-releasing factor consequent to lack of thymic action on hypothalamus development in perinatal life. Thymus implantation even at birth in nude mice does not modulate subsequent thyroxine levels when these animals reach maturity (Table 1). In our view, the failure to normalize the thyroid function by thymus implantation in newborn nude mice reflects the fact that the organization of the hypothalamus for thyroid function occurs earlier in ontogeny. This association between thymic and thyroid functions during foetal development could also be explained by the fact that both organs develop from the epithelium of the pharyngeal gut (Theiler, 1972). In addition, recent light and electron microscopic analysis has shown that the thymus rudiment in nude mice contains follicles morphologically very similar to those of the developing thyroid (Groscurth, Müntener & Töndury, 1974; Fukuda, Kistler & Groscurth, 1974).

There is also a body of evidence that it is the amount of testosterone during the neonatal period which leads to maturation of the brain–pituitary–gonad axis towards male or female sex differentiation and is responsible for onset of puberty and the ensuing cyclicity of reproductive functions in females (Young, 1961; Harris, 1964; Levine & Treiman, 1969; Jost, 1969; Jost *et al.*, 1973). In fact, recent work in this Institute has shown that puberty is considerably delayed in conventional and germ-free athymic and in neonatally thymectomized mice; thymus implantation normalizes its onset (Besedovsky & Sorkin, 1974).

Neonatally thymectomized and athymic adult female mice have lower levels of progesterone and 17-β-oestradiol (Tables 4 and 5). Implantation of thymus from newborn normal mice into newborn nude mice partially normalizes their sexual functions as shown by: (a) normalization of blood levels of oestradiol in females (Table 5); (b) by the regression of the transitory or X-zone in the adrenal cortex of athymic nude mice (Pierpaoli & Sorkin, 1972b); this zone is under the direct influence of gonadotropins (Jones, 1957); (c) by the normalization of time of puberty (Besedovsky & Sorkin, 1974). Levels of progesterone are not normalized by thymus implantation at birth (Table 4); the structural similarity between TSH and LH might account for these results (Pierce *et al.*, 1971). Further proof that the restoration of gonadal functions in athymic mice is only partial is provided by lack of 20-hydroxysteroid-dehydrogenase in the ovaries of adult female nude mice implanted with thymus at birth (Müller, E., personal communication). This enzyme appears during lysis of corpora lutea and catabolism of progesterone (Wiest & Kidwell, 1969). Finally, as is well known, thymus implantation at birth restores the capacity to reject allogeneic skin grafts. Here too a clear dissociation between the endocrine and the immune functions of the thymus is thus evident.

Our present findings suggest that the chronological influence of the thymus on the brain programming of certain gonadal functions (follicle-stimulating hormone and oestrogen synthesis) is extended for a few days in the postnatal time. It occurs later and requires less time than the programmation of thyroid function and other gonadal functions (LH and progesterone synthesis) which cannot be normalized by thymus implantation at birth. Six-day-old mice are immunologically still immature, irrespective of whether they are athymic or normal; therefore the likelihood that the observed endocrine anomalies reflect intercurrent infections or other such events is most unlikely.

A sharp increase in blood levels of corticosterone occurs in athymic mice at 14 days of age (Table 3). This finding might also be interpreted as a consequence of a permanent alteration in the mechanism of hypothalamic-pituitary regulation of synthesis and secretion of this steroid. In fact *in vitro* tests show that the adrenals of nude mice respond normally to ACTH stimulation and produce amounts of corticosteroids comparable to those of normal mice (Table 6).

Thyroid and ovaries of nude mice respond to injection of TSH and LH with normalization of levels of thyroxine and progesterone (Tables 7, 8 and 9). This shows that the alteration of thyroid and gonadal functions occurs primarily at the hypothalamic-pituitary level and not in the peripheral glands.

It is assumed that there are complex feed-back mechanisms which tend to compensate for endocrine alterations, but they are at present unknown. It seems a reasonable speculation that interference with their function could lead to a progressive deterioration of the endocrine system and thus bring about the severe 'wasting disease' that characterizes athymic and neonatally thymectomized mice. Thymus implantation itself prevents some of the endocrine

alterations; this effect cannot be duplicated by passive transfer of lymphocytes alone, an issue on which we have commented in previous work (Pierpaoli & Sorkin, 1972a).

It has long been considered that a major consequence of neonatal thymectomy (in mice and other species as well) is a wasting syndrome which primarily reflects an immune impairment.

There has also been substantial but less publicized work favouring a viral, microbial and toxin aetiology for wasting disease, based largely on the findings that athymic mice, or mice thymectomized at birth, raised in germ-free conditions do not develop this syndrome and their life span is prolonged (Rygaard, 1973; McIntire, Sell & Miller, 1964). These developments seemingly provide a logical explanation. However, there are data which prove that pathogenesis of the post-thymectomy wasting disease involves a good deal more than infection, but there is little doubt that the infection considerably speeds up its onset and course. For example, either neonatal thymectomy, or thymectomy as late as 4 weeks after birth, produces in the male hamster a typical wasting syndrome, despite the fact that this particular animal, at 4 weeks of age, is immunologically fully competent, as judged by antibody production and by rejection of allogeneic skin grafts (Sherman & Dameshek, 1964). This example also shows that the pathogenesis can be a sex-linked phenomenon.

The hormonal control of differentiation of sex obeys a completely chronologically asymmetrical pattern in both sexes. It is apparent that neonatal thymectomy in male hamsters greatly disturbs the mechanism of testosterone synthesis which in the neonatal stage affects the diversification of sex towards male. In fact, as we have shown (Table 2), the level of testosterone in athymic male mice is very low in the early postnatal age.

It is obvious that the kind of protection afforded by the germ-free environment is quite artificial and has not helped to define the real deficiency which, as previously postulated (Pierpaoli & Sorkin, 1972a), appears to be mainly endocrine. Therefore the deduction that the immune impairment is the cause of post-thymectomy wasting disease is a gross oversimplification of a quite different and much more complex syndrome. This once more attests to the dissociation and the simultaneous interdependence between the two functions of the thymus; the endocrine, and the immune which serves to generate a major category of immunocompetent cells.

The results of the present work thus point towards the thymus in mammals playing a crucial developmental role through its endocrine activity in early ontogeny which influences the future neuroendocrine performances of the host and eventually, as a consequence, the differentiation and function of thymus-derived lymphocytes (Pierpaoli *et al.*, 1970; Pierpaoli & Sorkin, 1972a). In this connection evidence has been adduced for endocrine deficits early in life in athymic and neonatally thymectomized mice. Some of these deficits, e.g. in sexual maturation, can be prevented by thymus implantation at birth. The present findings also provide data indicating a clear dissociation between the thymus influence on the immune status and on the neuroendocrine development, e.g. reconstitution of the immune function by administration of immunocompetent cells does not normalize the growth rate, life span and endocrine system of the animals and indicating that, even during the neonatal period (in which mice are immunologically immature) major shifts can be shown in blood hormonal levels of athymic mice. It is, however, not yet known whether the soluble factor(s) which are claimed to promote immunocompetence of lymphocytes (Davies & Carter, 1973) are related to or identical with the one(s) influencing neuroendocrine maturation. Indeed it seems possible that the real function of thymic factors is to promote a chronologically normal

development of the neuroendocrine system in early ontogeny and thereby create an environment in which the thymus- and bone marrow-derived precursor cells find the right conditions to become immunocompetent lymphocytes.

This participation of thymus factor(s) in the differentiation of the neuroendocrine system in early ontogeny and the role of the thymus in the establishment of the appropriate hormonal environmental conditions for the physiological maturation of the immune system have important implications for some of the most fundamental issues in immunobiology. Perhaps the overriding implication of the present findings is that the process of generation of immunological diversity and of recognition of self may well involve a sequential interaction of cells and hormones which, in turn, influence or perhaps even determine the immune and endocrine pattern of the individual. Preliminary findings based on still other immunological models seem to favour this hypothesis.

ACKNOWLEDGMENTS

We thank Miss Regula Kellerhals, Miss Katharina Naumann and Miss Ingrid Looser for skilful and devoted technical assistance. We are deeply grateful to Dr W. Rossbach, Institute for Biological and Medical Research of Hoffmann-La Roche & Company, AG, Füllinsdorf, for free supply of mice, and thank Dr H. Hurni and Miss M. Hämmerli, Animal Farm of Ciba-Geigy AG, Sisseln, for the experiments and observations carried out with germ-free mice. We thank Dr M. Landy for revising and correcting the manuscript. This work has been supported by the Swiss National Fund for Scientific Research, Grant number 3.8750.72, and the Emil Barell Foundation, Basel.

REFERENCES

ABRAHAM, G.E. (1969) Solid-phase radioimmunoassay of estradiol-17. *J. clin. Endocr.* **29,** 866.

ABRAHAM, G.E. (1974) Radioimmunoassay of steroids in biological materials. *Acta Endocr.* (*Kbh*), supplement **183,** 1.

ALEXANDER, D.P., BRITTON, H.G., FORSLING, M.L., NIXON, D.A. & RATCLIFFE, J.G. (1971) The concentrations of adrenocorticotrophin, vasopressin and oxytocin in the foetal and maternal plasma of the sheep in the latter half of gestation. *J. Endocr.* **49,** 179.

ANDERSON, D.C. (1970) A simple and clinically useful method for plasma testosterone-like substances by competitive protein binding. *Clin. chim. Acta.* **29,** 513.

BASSETT, J.M., THORBURN, G.D. & WALLACE, A.L.C. (1970) The plasma growth hormone concentration of the foetal lamb. *J. Endocr.* **48,** 251.

BESEDOVSKY, H. & SORKIN, E. (1974) Thymus involvement in female sexual maturation. *Nature* (*Lond.*), **249,** 356.

BIANCHI, E., PIERPAOLI, W. & SORKIN, E. (1971) Cytological changes in the mouse pituitary after neonatal thymectomy: a light and electron microscopical study. *J. Endocr.* **51,** 1.

BROWN-GRANT, K. & SHERWOOD, M.R. (1971) The 'early androgen syndrome' in the guinea pig. *J. Endocr.* **49,** 277.

BUUS, O. (1968) Double isotope technique for determination of the individual corticosteroids in human plasma. *Excerpta Med.* **157,** 107.

DAVIES, A.J.S. & CARTER, R.R.(1973) *Thymus Dependency. Contemporary Topics in Immunobiology.* Plenum Press, New York.

DEMETRIOUS, J.A. & AUSTIN, F.G. (1971) A rapid competitive protein-binding assay for plasma progesterone. *Clin. chim. Acta,* **33,** 21.

EAYRS, J.T. (1966) Thyroid and central nervous development. *Scientific Basis of Medicine Annual Reviews,* p. 317. Athlone Press, London.

FUKUDA, T., KISTLER, G. & GROSCURTH, P. (1974) The early postnatal development of the thymus in the nude mouse. II. Electron microscopic observations. *Proceedings of the 1st International Workshop on Nude Mice* (ed. by J. Rygaard and C. O. Povlsen), p. 37. Fischer Verlag, Stuttgart.

GOMEZ, C.J. (1971) Hormonal influences on the biochemical differentiation of the rat cerebral cortex. *Hormones in Development* (ed. by M. Hamburgh and E. J. W. Barrington), p. 417. Meredith Corporation, New York.

GROSCURTH, P., MÜNTENER, M. & TÖNDURY, G. (1974) The postnatal development of the thymus in the nude mouse. I. Light microscopic observations. *Proceedings of the 1st International Workshop on Nude Mice* (ed. by J. Rygaard and C. O. Povlsen), p. 31. Fischer Verlag, Stuttgart.

HARRIS, G.W. (1964) Sex hormones, brain development and brain function. The Upjohn Lecture of the Endocrine Society. *Endocrinology*, **75,** 627.

JONES, K.C. (1957) *The Adrenal Cortex*. Cambridge University Press, Cambridge.

JOST, A. (1969) The extent of foetal endocrine autonomy. *Foetal Autonomy. Ciba Foundation Symposium* (ed. by G. E. W. Wolstenholme and M. O'Connor), p. 79. Churchill, London.

JOST, A., VIGIER, B., PREPIN, J. & PERCHELLET, J.P. (1973) Studies on sex differentiation in mammals. *Rec. Progr. Hormone Res.* **29,** 1.

KLIMAN, B. & PETERSON, R.E. (1960) Double isotope derivative assay of aldosterone in biological extracts. *J. biol. Chem.*, **235,** 1639.

LEVINE, S. & TREIMAN, L.J. (1969) Role of hormones in programming the central nervous system. *Foetal Autonomy. Ciba Foundation Symposium* (ed. by G. E. W. Wolstenholme and M. O'Connor), p. 271. Churchill, London.

MCINTIRE, K.R., SELL, S. & MILLER, J.F.A.P. (1964) Pathogenesis of the post-neonatal thymectomy wasting syndrome. *Nature (Lond.)*, **204,** 151.

MIKHAIL, G., WU, C.H., FERIN, M. & VAN DE WIELE, R.L. (1970) Radioimmunoassay of plasma estrone and estradiol. *Steroids*, **15,** 333.

MUELLER, J. (1965) Aldosterone stimulation *in vitro*. I. Evaluation of assay procedure and determination of aldosterone stimulation activity in a human urine extract. *Acta Endocr. (Kbh)*, **48,** 283.

MURPHY, B.E.P. & JACHAN, C. (1965) The determination of thyroxine by competitive protein-binding analysis employing an anion exchange resin and radiothyroxine. *J. Lab. clin. Med.* **66,** 161.

MURPHY, B.E.P., PATTEE, C.J. & GOLD, A. (1966) Clinical evaluation of a new method for the determination of serum thyroxine. *J. clin. Endocr.* **26,** 247.

PIERCE, J.G., LIAO, T., HOWARD, S.M., SHOME, B. & CORNELL, J.S. (1971) Studies on the structure of thyrotropin: its relationship to luteinizing hormone. *Rec. Progr. Hormone Res.* **27,** 165.

PIERPAOLI, W. & SORKIN, E. (1967) Relationship between thymus and hypophysis. *Nature (Lond.)*, **216,** 834.

PIERPAOLI, W., FABRIS, N. & SORKIN, E. (1970) Developmental hormones and immunological maturation. *Hormones and the Immune Response. Ciba Foundation Study Group No.* 36 (ed. by G. E. W. Wolstenholme and J. Knight), p. 126. Churchill, London.

PIERPAOLI, W. & SORKIN, E. (1972a) Hormones, thymus and lymphocyte functions. *Experientia (Basel)*, **28,** 1385.

PIERPAOLI, W. & SORKIN, E. (1972b) Alterations of adrenal cortex and thyroid in mice with congenital absence of the thymus. *Nature: New Biology*, **238,** 282.

RYGAARD, J. (1973) Immunobiology of the mouse mutant nude. *Thymus and Self.* F.A.D.L., Copenhagen.

SHERMAN, J.D. & DAMESHEK, W. (1964) Post-thymectomy wasting disease of the golden hamster. *The Thymus in Immunobiology*, (ed. by R. A. Good and A. E. Gabrielsen), p. 542. Harper & Row, New York.

SOLOMON, J.B. (1971) *Foetal and Neonatal Immunology*. North-Holland Publishing Company, Amsterdam.

THEILER, K. (1972) Development and normal stages from fertilization to 4 weeks of age. *The House Mouse*. Springer, Berlin.

UETTWILLER, A. (1970) Testosteron Bestimmung im Plasma und Urin mittels 'competitive protein binding method'. *Z. Klin. Chem. und Klin. Biochem.* **8,** 225.

WIEST, W.G. & KIDWELL, W.R. (1969) The regulation of progesterone secretion by ovarian dehydrogenases. *The Gonads* (ed. by K. W. McKerns), p. 295. North-Holland Publishing Company, Amsterdam.

YOUNG, W.C. (1961) The hormones and mating behaviour. *Sex and Internal Secretions* (ed. by W. C. Young), p. 1173. Bailliere, Tindall, London.