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HORMONES AND THE IMMUNOLOGICAL CAPACITY

IV. RESTORATIVE EFFECTS OF DEVELOPMENTAL HORMONES OR OF LYMPHOCYTES ON THE IMMUNODEFICIENCY SYNDROME OF THE DWARF MOUSE

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SUMMARY

The restorative action of developmental hormones, mainly somatotropic hormone, and of lymphnode lymphocytes on the immunologically crippled hypopituitary Snell-Bagg dwarf mouse is evaluated. The hormonal treatment can completely reconstitute the structure of the thymus and of the peripheral lymphoid tissues. Both hormones and mature lymphocytes restore the impaired capacity of the dwarf mouse to propuce antibody and to reject skin allografts. Normal donor thymocytes and bone marrow cells alone or in combination fail to produce the same effect in absence of a hormonal treatment. The action of hormones is not exerted if dwarf mice are thymectomized in adult age. This and other evidence shows that the action of hormones is mediated through the thymus and leads to the formation of long-living lymphocytes.

INTRODUCTION

As reported in the preceding paper (Fabris, Pierpaoli & Sorkin, 1971) dwarf mice are affected by a thymus-dependent immunodeficiency disease. The aetiology of this syndrome originates in a pituitary disorder with quantitative deficiencies of synthesis of somatotropic and thyrotropic hormone. Previous findings (Pierpaoli *et al.*, 1969) have shown that treatment of dwarf mice with somatotropic hormone and thyroxine results in reconstitution of the immune capacity and restoration of the histological structure and cell population of lymphatic tissues.

The main purpose of this paper is to define the extent and the kinetics of such a hormonal reconstitution. The immunological parameters studied were the number of peripheral blood lymphocytes, size and structure of thymus and spleen, the primary humoral immune response to sheep red blood cells and the survival of allogeneic skin-grafts. In order to

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evaluate which lymphoid cells are deficient in dwarf mice and are produced during the hormonal treatment, experiments were performed to reconstitute the immunodeficiency of dwarf mice by injection of different lymphoid cell populations, such as thymocytes, peripheral lymphocytes and bone marrow cells. Finally, the failure to reconstitute dwarf mice after adult thymectomy with hormones will be discussed in relation to the immunological function of the thymus during postnatal life and its hormonal control.

MATERIALS AND METHODS

Animals. Dwarf and normal Snell-Bagg mice were used. The origin and maintenance of these mice have been described in the preceding paper (Fabris, Pierpaali & Sorkin, 1971).

Histological and immunological techniques. See preceding paper.

Hormone preparations. Bovine growth hormone (NIH-GH-B15, 0·88 USP unit/mg), was kindly supplied by Dr Wilhelmi, through the courtesy of Dr Graaf, Endocrinological Study Section, NIH, Bethesda, Maryland, U.S.A. Bovine somatotropic hormone (STH) Raben type (1 USP unit/mg), was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. For injection the solutions were prepared freshly by dissolving the hormone in alkalinized physiological saline, pH 7·8–8·0, and injected subcutaneously at a daily dose of 250 μg. Bovine luteotropic hormone (NIH-P-B3 24·1 I.U./mg) was kindly supplied by the Endocrinological Study Section, NIH. The hormone was dissolved in physiological saline and injected at a daily dose of 250 μg. Bovine thyrotropic hormone (NIH-TSH-B4 2·21 USP u/mg) was kindly supplied by the Endocrinological Study Section, NIH. It was dissolved in physiological saline and injected at a daily dose of 40 μg. L-Thyroxine (Hoffmann–La Roche, Basel, Switzerland) was dissolved in alkalinized physiological saline and injected at a daily dose of 1 μg. Insulin (40 I.U./ml), Novo Industri A/S, Copenhagen, Denmark, was diluted in physiological saline and injected at a daily dose of 0·016 I.U.

Preparation of cell suspensions

Thymus lobes were removed from 4–5-week-old mice, care being taken to avoid fascial tags which might contain lymphnodes. The organs were teased by fine forceps in cold Gey's medium. Further disruption was achieved by gentle aspiration with a Pasteur pipette. After sedimentation for 10 min the supernatant was centrifuged and the cells washed with Gey's solution. Cells were finally resuspended, counted and the volume adjusted so that the number of cells required for injection was contained in 0·2 ml. Peripheral lymphnode cells were obtained from axillary, inguinal and mesenteric lymphnodes by teasing, washing and resuspending as mentioned above. Bone marrow cells were obtained from the femurs and the tibiae by washing them out with a syringe containing cold Gey's medium. The plugs were disrupted by aspiration through a 25 gauge needle. The suspension was washed once and resuspended as above.

Injections. Unless otherwise stated, hormonal reconstitution commenced between 20 and 30 days of age and was continued for 20 days. Cell suspensions were injected i.p.

Operative procedures. Thymectomy or sham-operation was performed in newborn mice, not later than 24 hr after birth. Adult-thymectomy or sham-operation was performed on 4–5-week-old animals. Whenever the operated animals were killed, the mediastinum was carefully controlled for macroscopical thymus remnants.

RESULTS

(A) *The restorative effect of developmental hormones on the immunodeficiency syndrome in dwarf mice*

The alterations of endocrine function of dwarf mice, as reported in the preceding paper, are mainly represented by deficiencies in pituitary STH, TTH and LTH synthesis, with concomitant decrease in ACTH and gonadotrophin production. As a consequence of these alterations some target glands of the hypophysis are underdeveloped. Therefore also deficiencies of thyroxine, adrenal and gonadal steroids, and of pancreas hormones are more or less present in dwarf mice. In order to test which of these hormones are responsible for the immunological disorders in dwarf mice, the different hormones were injected individually to evaluate their capacity to overcome the immunodeficiencies.

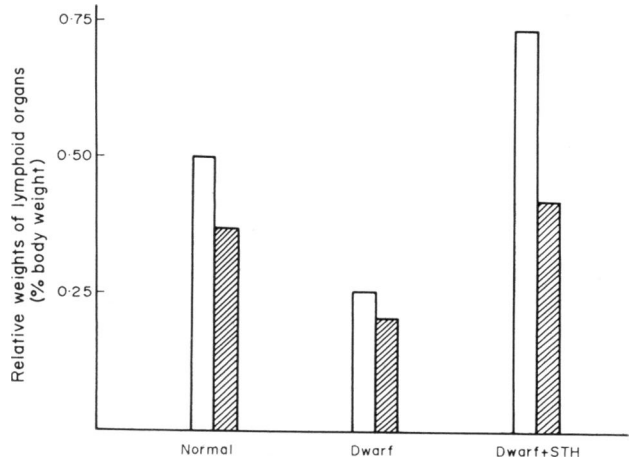

FIG. 1 Increase of thymus (hatched columns) and spleen (open columns) relative weights in Snell-Bagg dwarf mice after 20 days treatment with 250 μg STH.

Our main attention has been concentrated on somatotropic hormone (STH), because its deficiency is the most pronounced and of greatest consequence in the dwarf mice. Therefore the effect of this hormone on the immune system will be reported in more details, and the effects of other hormones are compared with those of STH.

(1) *Influence of STH on thymus*

As already reported (Pierpaoli *et al.*, 1969) 10 days treatment with STH and Tx reconstitutes the histological structure of the thymus of dwarf mice. A similar recovery is obtained treating dwarf mice with STH only. When treatment is prolonged for 20 days (as done in the usual reconstitution experiments) both the absolute and relative weights of the thymus of the dwarf mice are increased (Fig. 1). Particularly, the relative weights reach the values of normal littermates and are in fact frequently even higher. Absolute thymus weights do not reach normal values simply because the hormonal treatment is never able to reconstitute completely body growth. Histologically the thickness of the thymus cortex is increased and shows repopulation with small lymphocytes.

(2) *STH influences the peripheral lymphoid tissues*

As reported in the previous paper, spleens of dwarf mice are hypotrophic, particularly in thymus-dependent areas. Hormonal reconstitution with STH increases both absolute and relative spleen weights in dwarf mice (Fig. 1). Histologically lymphoid follicles are increased in number and size. In particular the periarteriollar sheats and the parafollicular areas of lymphoid follicles are fully repopulated with small lymphocytes; the thickness of these areas is frequently higher than in normal animals of the same age (Fig. 2). Similar patterns of complete recovery were observed in the peripheral lymphnodes of dwarf mice after hormonal treatment. Plasma cells, both in the red pulp of the spleen and in the medullary cords of lymphnodes, increased under STH influence and frequently reached values over the normal level.

(3) *STH influences the bone marrow*

As already reported (Martinazzi & Giraldi, 1962), reconstitution of all kinds of bone marrow cells occurs after hormonal treatment. Differential counts did not reveal specific effects of STH treatment on the different cellular components of bone marrow.

(4) *STH influences peripheral blood lymphocytes*

As shown in Fig. 3, PWBC, usually low in dwarf mice, increase when STH is given daily; twenty-five injections are needed to reach normal PWBC level, when hormonal treatment begins at 30 days of age. The reconstitution effect is mainly concerning peripheral blood lymphocytes, while polymorphonucleated cells are only slightly augmented (Fig. 4). If hormonal treatment is interrupted after ten daily injections, the increase in PWBC number does not stop immediately but shows a progressive arrest during the following days. Such a phenomenon is present also when treatment is interrupted after twenty-five daily injections (Fig. 3). The cause for these facts is unknown.

(5) *The effect of STH on primary immune response to SRBC*

The deficient primary humoral immune response against SRBC, as measured by PFC capacity 4 days after immunization, is reconstituted by treating dwarf mice with STH and Tx for 20 days (Pierpaoli *et al.*, 1969). However, reconstitution occurs also when dwarf mice are treated with STH alone for 20 days (Fig. 5). The kinetic studies on the primary immune response support the view that in fact such a recovery consists in a prevention of the delay in the onset of the response. However, this reconstitution can take place not only when SRBC are injected during the hormonal treatment, as it was done in our earlier experiments, but also when SRBC are injected some weeks after hormonal reconstitution (Fig. 5). In contrast, if hormonal treatment begins on the day of antigen injection, STH is unable to significantly prevent the observed delay, but in the period following the first week after challenge, STH increases the quantity of the response to normal levels.

(6) *STH treatment shortens skin-graft survival*

As shown in Fig. 6, the survival of allogeneic skin-grafts, which is prolonged in dwarf mice, is shortened to normal values by treatment with STH, when the daily hormone injections started 7 days before grafting and continued until rejection occurred. Also the local processes seem to be accelerated.

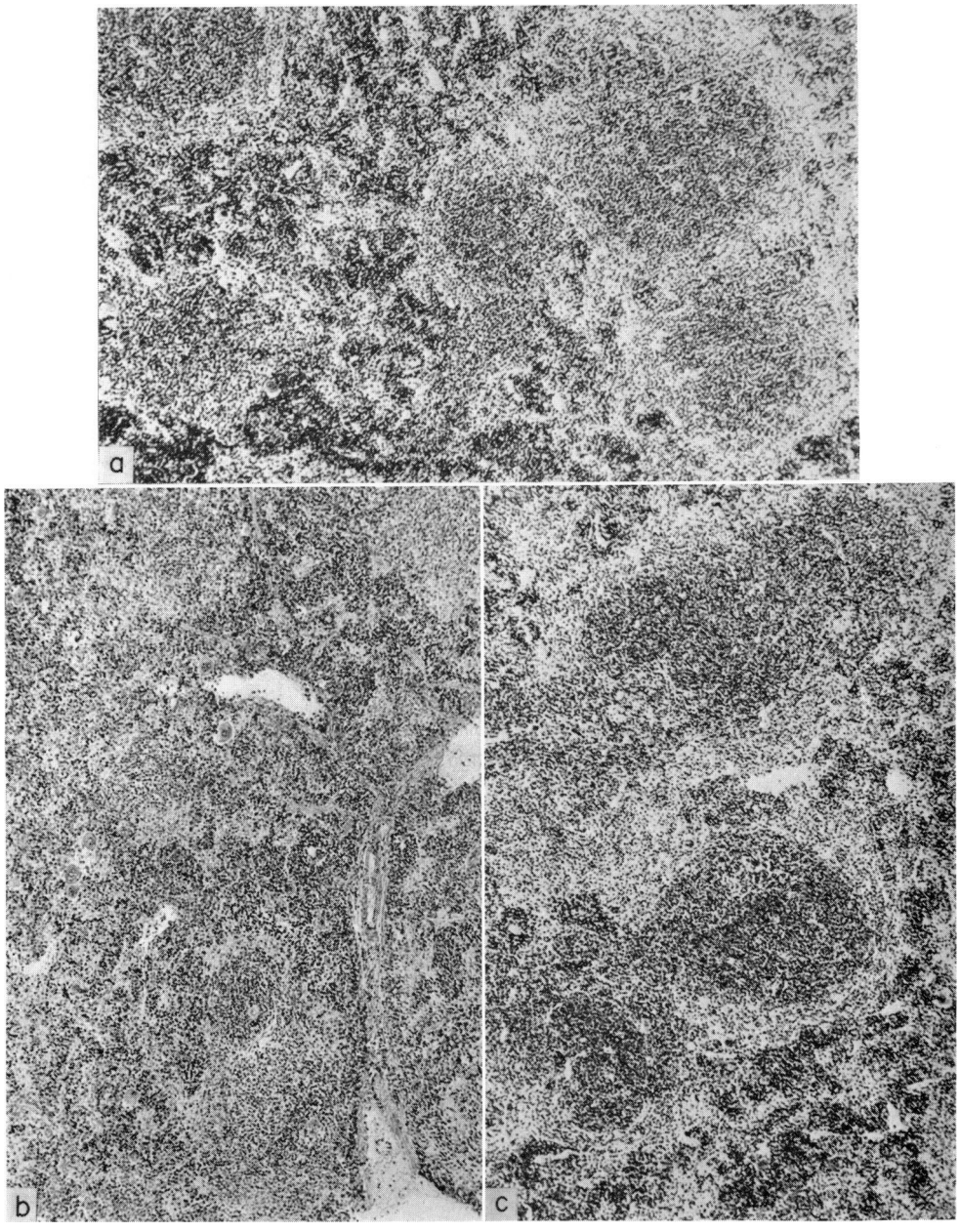

FIG. 2. Histological sections of spleens from Snell-Bagg mice. (a) Normal animal; (b) untreated dwarf mouse (note the reduction in number of lymphoid cells in the white pulp); (c) dwarf mouse treated with 250 μg STH for 20 days (note the complete reconstitution of the spleen structure). Haematoxylin–eosin, × 63.

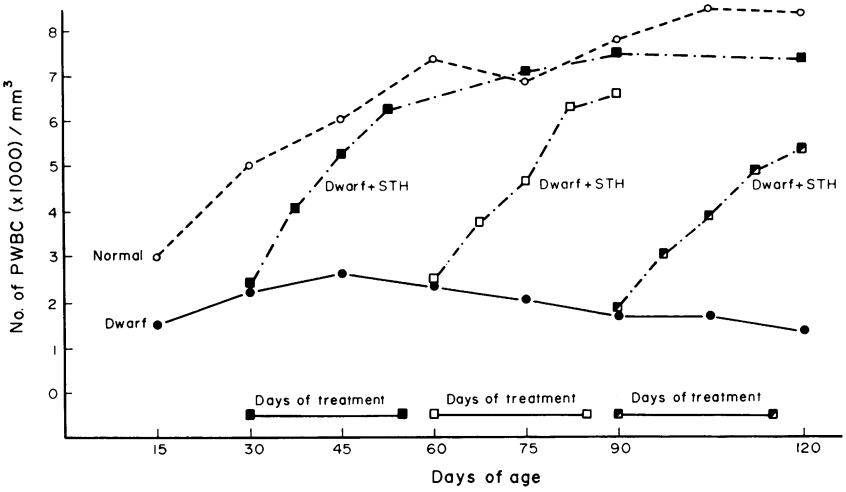

FIG. 3. Influence of 25 days of treatment with 250 μg STH on number of PWBC. Note maintenance of number of PWBC after termination of STH treatment in the group treated from day 30 of age.

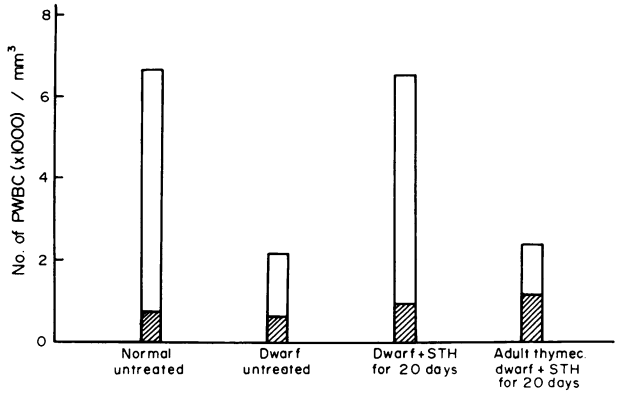

FIG. 4. Influence of twenty daily injections with 250 μg STH on peripheral lymphocytes (open columns) and polymorphonucleated cells (hatched columns) of non-operated and adult thymectomized dwarf mice.

(7) *STH treatment increases the life span of dwarf mice*

The usual STH reconstitution therapy (twenty daily injections) is able to prolong the life-span of dwarf mice from 100 to 300–400 days. Details will be published elsewhere.

(8) *Influence of STH on body growth*

Besides the restorative effects of STH on immunological parameters in dwarf mice, this hormone produces, as expected, also a pronounced increase in body growth during its application. Withdrawal of STH results in immediate termination of growth. It is of interest to note that while one injection of lymphocytes can also restore the immunological capacity, no concomitant body growth occurs.

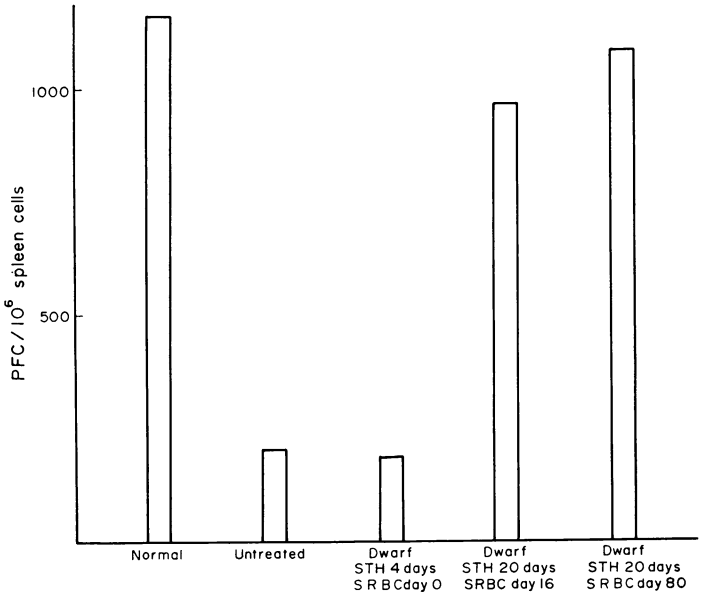

FIG. 5. Long-lasting reconstitution of immune capacity of dwarf mice by STH treatment. Dose of SRBC: 4×10^8. Daily dose of STH: 250 μg.

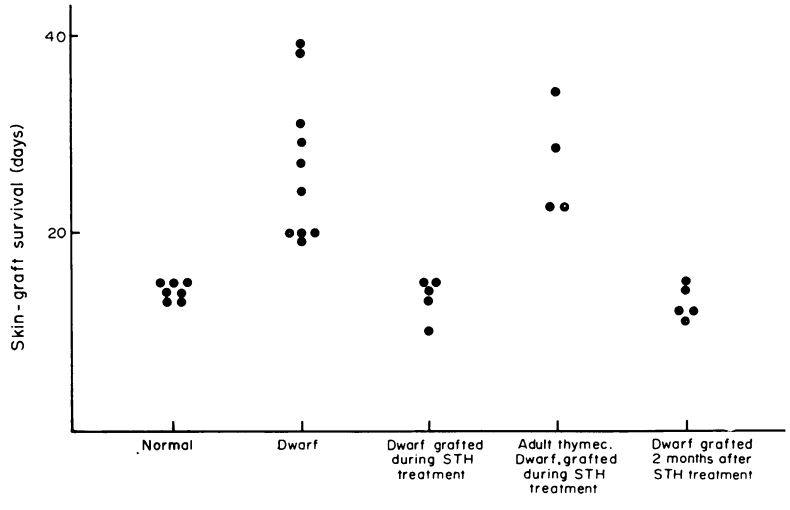

FIG. 6. Thymus-dependent and long-lasting reconstitution of homograft immunity in dwarf mice after daily treatment with 250 μg STH. STH treatment was begun 7 days before transplantation and continued until rejection occurred. Thymectomy was performed on 30-day-old dwarf mice; skin-grafts were performed 10 days after the operation.

(9) *Influence of other hormones on dwarf mice*

Among the other hormones, whose synthesis is reduced in dwarf mice, many have been already tested for their capacity to restore PWBC number and spleen structure (Martinazzi & Giraldi, 1962). It has been established by hormonal replacement therapy that the histologically documented immunodeficiency of dwarf mice cannot be overcome by ACTH, by follicle stimulating hormone or by luteinizing hormone.

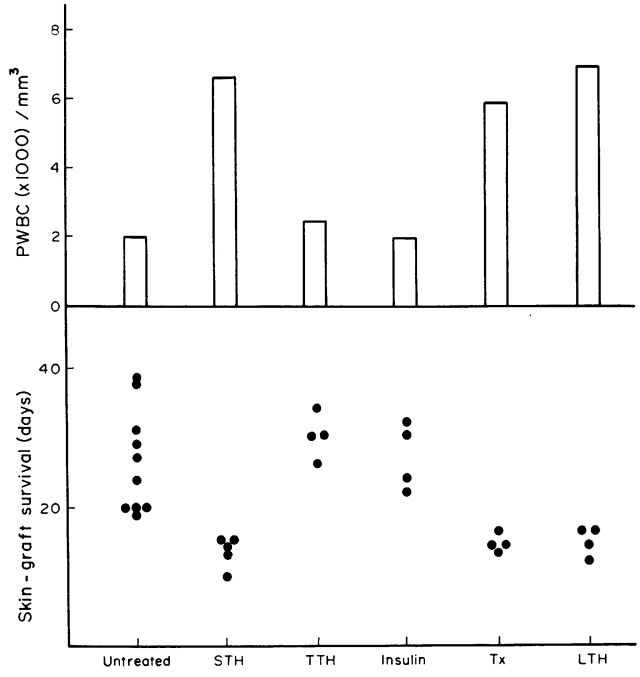

FIG. 7. Effect of different hormones on PWBC number and survival of allogeneic skin-grafts in dwarf mice. The evaluation of PWBC number was performed after twenty-five daily injections of the different hormones. In order to test for skin-graft survival, animals received seven daily injections with hormones before grafting and the treatment was then continued until rejection.

Therefore neither these hormones nor the hormones produced by their target glands, such as adrenal and gonadal steroids, are involved in the immunodeficiency of dwarf mice. Also TTH or insulin do not affect PWBC numbers and skin graft survival as is shown in Fig. 7. In contrast Tx, LTH and the combination STH and Tx are able to overcome the immunodeficiencies of dwarf mice.

(B) *Thymus-dependency of the hormonal action*

In order to test whether the immunological recovery of dwarf mice under hormonal treatment is due to a peripheral effect or is mediated through the thymus, dwarf mice were thymectomized at 30 days of age and then treated with STH for 20 days or longer. As shown in Fig. 4, PWBC number does not increase under STH influence if the thymus is not present. In contrast normalization of PWBC is obtained with the same hormonal treatment in non-

thymectomized dwarf mice. Also the delayed skin-graft rejection in dwarf mice cannot be accelerated by STH treatment in the absence of the thymus (Fig. 6).

Finally neither the PFC capacity 4 days after immunization nor the reduced size and abnormal structure of the spleen can be reconstituted by injecting STH into adult thymectomized dwarf mice, this in spite of the good body growth response to STH. Similar results were obtained using neonatally thymectomized dwarf mice. In agreement with our previous work (Fabris, Pierpaoli & Sorkin, 1970) we observed that hormonal therapy was unable either to prevent wasting disease or to delay its onset. On the other hand the hormonal deficiency of dwarf mice did not induce an earlier onset of the wasting disease showing that the pituitary hormones are probably acting through the thymus.

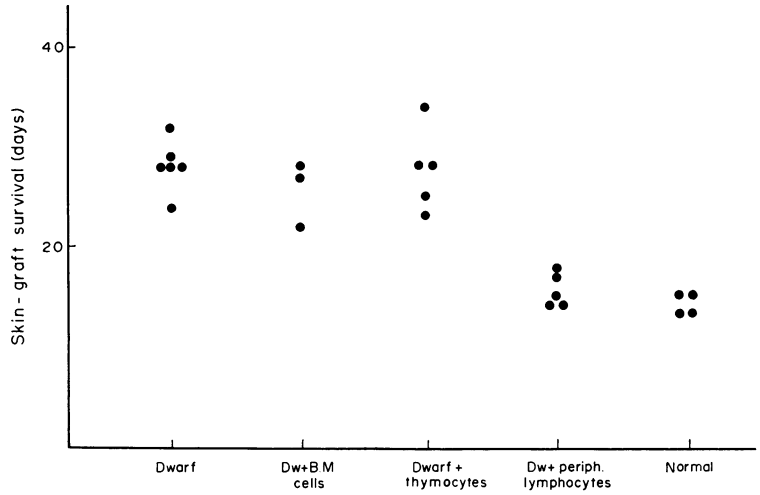

FIG. 8. Restorative effects of one i.p. injection with 150×10^6 lymphnode lymphocytes on skin-graft survival in dwarf mice. Equal amounts of bone marrow cells or thymocytes were also injected in other groups. Skin from Charles River donors was grafted 1 day after injection of the cells. Cell donors: 4–5-week-old normal Snell-Bagg mice.

(C) *The restorative effect of lymphnode cells*

In order to test which lymphoid cell population is increased in dwarf mice during the hormonal treatment, replacement therapy with lymphoid cells of different origin was carried out. Groups of dwarf mice were injected i.p. either with 150×10^6 lymphnode lymphocytes or with equal amounts of thymocytes or bone marrow cells from 4–5-week-old normal littermate donors. The immunocompetence of dwarf mice was evaluated by allogeneic skin-graft rejection and primary immune response to SRBC.

(1) *Lymphnode lymphocytes shorten skin-graft survival.* Skin from Charles River donors was grafted the day after the injection of lymphoid cells and its survival time was evaluated. As shown in Fig. 8, injection of bone marrow cells or thymocytes is unable to induce in dwarf mice the same restorative effects as hormonal reconstitution, while injection of lymphnode lymphocytes shortens the skin-graft survival time without any hormone therapy.

(2) *Lymphnode lymphocytes restore the humoral immune response to SRBC.* Groups of dwarf mice were injected with 150×10^6 lymphnode lymphocytes or with equal amounts of

thymocytes alone or together with 50×10^6 bone marrow cells. At the same time all animals were challenged i.p. with SRBC. Other groups were injected either with lymphnode lymphocytes or thymocytes as reported above, but injection with bone marrow cells and SRBC took place 20 days later. In both experimental conditions only lymphnode lymphocytes, as shown in Fig. 9, are able to significantly increase the haemaglutinin titres in dwarf mice, while the injection of thymocytes or bone marrow cells alone, or in combination, was ineffective. From these experiments we can conclude that dwarf mice do not show significant defects in the behaviour of bone marrow cells.

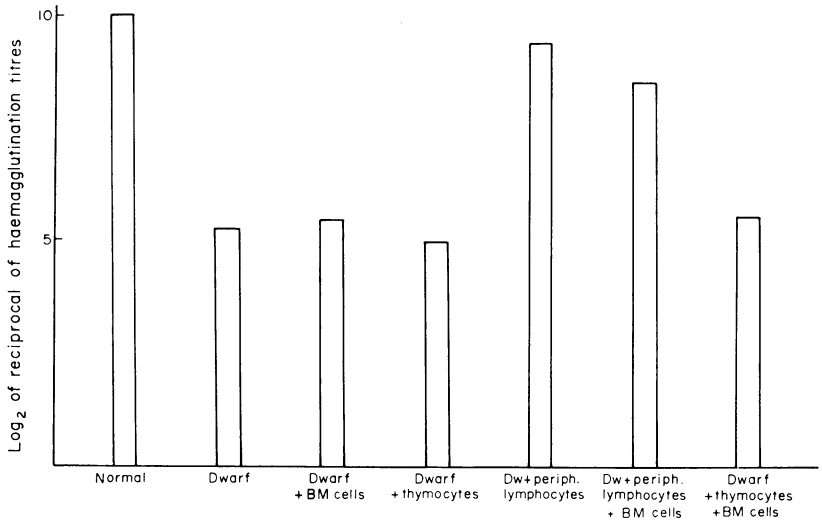

FIG. 9. Restorative effects of one i.p. injection with 150×10^6 lymphnode lymphocytes on haemagglutinin formation in dwarf mice. Antibodies were measured 4 days after i.p. injection of 4×10^8 SRBC. Dose of thymocytes: 150×10^6. Dose of bone marrow cells: 50×10^6. Lymphoid cells and the antigen were injected simultaneously. Cell donors: 4–5-week-old normal Snell-Bagg mice.

(D) *The long-lasting effect of hormonal reconstitution in dwarf mice*

The complete recovery of the immunodeficiencies present in 25-day-old dwarf mice is achieved by 20 days treatment with STH. The problem, whether such a recovery is transitory or persists long after hormone therapy was discontinued, was investigated on groups of dwarf mice which were hormonally reconstituted between 25 and 45 days of age and then left without any further treatment. Two or 3 months later their PWBC number, their capacity to reject skin-grafts and to form plaques 4 days after immunization with SRBC was evaluated. The absence of further hormonal treatment after reconstitution is neither followed by a decrease in PWBC number (Fig. 3) nor by a disappearance of cell-mediated (Fig. 6) or humoral immune response (Fig. 5). The long-lasting kind of immunological recovery which follows hormonal treatment of dwarf mice is also confirmed by the prolongation of their life-span, as will be reported elsewhere.

Similar long-lasting effects have been observed after treatment of dwarf mice with peripheral lymphnode lymphocytes. However, no complete experimental evaluation was performed to compare the effect of lymphnode lymphocytes with the effects of other types of lymphoid cells.

(E) *Hormonal sensitivity of the immune system of dwarf mice at different ages*

The presence of lymphoid cells which are directly or indirectly sensitive to STH at 20–30 days of age has been proven by the reconstitution experiments reported above. In order to test whether such a sensitivity is present also in later periods of life or whether it is disappearing, experiments have been performed to reconstitute 60- or 90-day-old dwarf mice with STH. As immunological parameters PWBC number, skin graft survival and PFCs 4 days after immunization were evaluated. As shown in Fig. 3, PWBC number can be reconstituted starting the treatment with STH at 60 or 90 days of age. The speed of the PWBC recovery does not show significant differences among the different age groups tested. However, the time needed for complete reconstitution of PWBC number by hormonal therapy is slightly increased in 90-day-old dwarf mice as compared to 30-day-old animals. No differences were observed in skin-graft survival time or in PFC capacity after hormonal treatment in the first two age groups (our unpublished results).

DISCUSSION

The experiments reported above using the hypopituitary immunodeficient dwarf mouse have served as an experimental model to evaluate the relation between endocrinological and immunological functions. We wish to discuss here the conclusions and implications drawn from these experiments.

(1) *Developmental hormones, mainly STH, restore the deficient immune system of dwarf mice*

This conclusion is based on two facts:

(a) The main endocrine deficiency of dwarf mice involves the developmental hormones, i.e. somatotropic hormone, lactotropic hormone and thyroxine.

(b) All the deficient immunological functions in dwarf mice can be restored to physiological levels by treatment with the developmental hormones. Other hormones such as TTH, insulin, adrenal and gonadal steroids are ineffective.

There are various interpretations of the mode of the restorative action of the developmental hormones. It is known that some effects of hormones are due to the metabolic alterations induced by them. Indirect observations are clearly supporting the idea that metabolic consequences of the hormonal therapy are not responsible for the effects on the immune system in our model. Thus the logarithmic increase of PFCs after primary antigenic stimulation with SRBC (Fig. 6, preceding paper) and the immunoglobulin levels are normal in dwarf mice (Wilkinson, Singh & Sorkin, 1970) whereas cell-mediated immunity or PWBC number are deficient. Furthermore dwarf mice can be reconstituted in their immunological capacity by injection of adult lymphocytes without apparently influencing the metabolic disorders. Finally the hormonal deficiency is not affecting all tissues in dwarf mice, e.g. the turnover of DNA or RNA in the tongue or in submaxillary glands is normal while it is extremely reduced in thymus and spleen (Winick & Grant, 1968).

These indirect observations support the view that the effects of developmental hormones on the immune system of dwarf mice are due to their direct action on some hormone-sensitive lymphoid cells. Such a sensitivity has also a certain degree of specificity as is confirmed by the fact that not all lymphoid tissues are equally sensitive to hormones. In our experimental model it is primarily the thymus which contains hormone-sensitive cells (see section 2 and 3 of Discussion).

We have observed that reconstitution of dwarf mice is possible with the developmental hormones STH, LTH and thyroxine. The problem is to define which of them has a direct and physiological action on the development of the immune system of the dwarf mice. Prolactin can be excluded because it is present in negligible amounts in males. Its effect can best be attributed to the similarity between LTH and STH.

The action of thyroxine is unexplained. We favour the view that thyroxine causes increased synthesis and release of STH from the few STH-producing cells present in the hypophysis of dwarf mice (Solomon & Greep, 1959). Also the possibility of a synergistic action of Tx with small amounts of STH cannot be discarded.

Even if it is considered that STH is the main hormone specifically acting on the proliferation of the immune system in dwarf mice, the possibility still exists that STH acts in cooperation with some non-developmental hormones, as insulin, or as antagonizing others, as corticosteroids.

ACTH synthesis is decreased and consequently adrenals are underdeveloped. Also if a relatively high level of corticosteroids might be responsible for lymphoid tissue involution in dwarfs, this does not diminish the importance of STH because it has been demonstrated that STH can antagonize the cortisol-induced suppression of antibody formation (Fabris, Pierpaoli & Sorkin, 1970).

Insulin also needs to be considered in relation to STH action (Young, 1968). Although insulin has no effects in our experimental model, this does not exclude that the amount normally available in dwarf mice is high enough to act in synergism with STH for the development of the immune system.

(2) *The action of STH is mediated through the thymus*

This proposition is based on the failure to restore the immunodeficiencies of dwarf mice with STH, if the thymus has been removed previously. Neither the diminished peripheral lymphocytes count (Fig. 4) nor the graft survival time (Fig. 6), can be reconstituted in adult thymectomized dwarf mice, even if hormonal treatment is prolonged for more than 20 days. Previous observations, besides those presented here, fully agree with our proposition (Pierpaoli, Fabris & Sorkin, 1970) that the thymus contains hormone-sensitive cells. For the mode of action of STH on the thymus several explanations can be offered: (a) STH induces some thymic precursors, presumably bone marrow cells, to proliferate and/or to differentiate to immunocompetent lymphocytes. (b) STH acts on cells, to induce them to synthesize a thymus humoral factor, which might act not only within the thymus but also at the periphery (Trainin & Small, 1970; White & Goldstein, 1970). While our data do not allow us to favour one or the other explanation, they permit the conclusion that the relation between STH and thymocytes is specific.

(3) *The result of STH action on the thymus is the immunocompetent lymphocyte*

Among the effects of STH therapy on the immune system of dwarf mice, the most clear-

cut seem to be the induced growth of the thymus, the recovery of peripheral blood lymphocytes and the reduced skin-graft survival time. The experiments shown in Figs 8 and 9 have proven that lymphnode lymphocytes can reconstitute dwarf mice to a similar extent as STH. Therefore we may presume that the action of STH is to increase the population of thymocytes and immunocompetent cells. Whether this effect is due to differentiation of thymic precursors into immunocompetent cells or to the proliferation of pre-existing immunocompetent lymphocytes which might be present in the thymus is not possible to decide.

(4) *The influence of STH on the thymus is present during the whole life*

If we assume that the main action of STH is to induce the proliferation of lymphoid cells within the thymus to give a mature population of immunocompetent lymphocytes, it seems reasonable to suppose that such action is exerted throughout life. Although the dwarf mice are not the best experimental model because of their limited life-span, the immunological reconstitution obtained with STH treatment on 60- or 90-day-old dwarf mice is suggesting that at least until this age STH-sensitive cells are present.

However, the removal of the hypophysis in young adult rats does not induce any immediate peripheral defects in the humoral and cell-mediated immune responses (Kalden, Evans & Irvine, 1970). These findings have been considered to be in disagreement with the results obtained in the dwarf mice.

This discrepancy is not a fundamental one because the effect of STH in dwarf mouse is to increase to normal level the deficient number of lymphocytes, which thereafter can maintain their immune capacity for a long period of time, as proven by the long-lasting kind of reconstitution in the dwarf mice. In contrast, in the hypophysectomized rats such lymphocytes have been already formed in normal amount before the time of operation, and their long-lasting immunocompetence prevents the animals showing any immediate peripheral defect. Therefore the action of STH can be experimentally best observed in normal animals when the immune system is not completely developed, or when it has been artificially destroyed by cortisonization or X-irradiation.

The quality of the effect of STH does not seem to differ in early postnatal or in adult age, but the requirement for STH will naturally be higher during the ontogenetic formation of immunocompetent cells in early life (Pierpaoli, Fabris & Sorkin, 1970).

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ABBREVIATIONS

STH	somatotropic hormone
Tx	thyroxine
LTH	luteotropic hormone
TTH	thyrotropic hormone