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Immunological Studies on the Rat Thymectomized in Adult Life

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Summary. Rats thymectomized in adult life showed no decrease in ability to produce circulating antibodies to sheep erythrocytes and tetanus toxoid even when tested 270 days after thymectomy. From 120 days after thymectomy onwards allogeneic skin-graft rejection times were increased to about twice that found in control animals.

Rabbit anti-rat lymphocyte serum (ALS) was given to non-thymectomized rats and, at varying intervals after thymectomy, to rats which had been thymectomized in adult life. Non-thymectomized rats so treated showed no decrease in their ability to reject allogeneic skin grafts or to produce antibodies to sheep erythrocytes. In contrast there was a significant depression of the immune response of all thymectomized rats given ALS. Allogeneic skin graft rejection times were longer in animals given ALS 180 and 240 days after thymectomy than in any of the other groups of animals.

There was a significant depression in the ability of animals given ALS 240 days after thymectomy to produce antibody in response to the second of two doses of tetanus toxoid injected 7 weeks after the administration of ALS. All other animals behaved normally when immunized in this way.

Miller (1963) has suggested that there are in the marrow cells with immunological potential which can only express this potential when the thymus is present. This hypothesis implies that after an animal has been thymectomized in adult life it will become incapable of producing new immunologically competent cells; that is, cells capable of producing antibody when stimulated by antigen. Immunologically competent cells present at the time of thymectomy will presumably retain their competence. It is however possible that immunologically competent cells which have not become committed by exposure to antigen normally die and disappear with the passage of time. This process, if it occurs, should result in a gradual decrease in the ability of a rat thymectomized in adult life to produce an immune response.

It is possible that most of the immunologically competent cells possessed by a rat belong to the lymphocyte family of cells. The administration of a rabbit anti-rat lymphocyte serum (hereafter referred to as ALS) to a rat thymectomized in adult life should result in the destruction of any immunologically competent lymphocytes present in these animals at the time of thymectomy and persisting after it. This should result in a depression of the immune response of animals so treated; that is, if the rat thymectomized in adult life is unable to produce new generations of immunologically competent lymphocytes.

In this investigation the immune response of thymectomized rats has been studied at varying intervals after thymectomy and also after the administration of ALS. The results obtained support the validity of Miller's hypothesis and its implications as discussed above.

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MATERIALS AND METHODS

Plan of experiments

Female Sprague-Dawley rats were thymectomized when 12 weeks old and groups of these animals were tested at 0, 60, 120, 180 and 240 days after thymectomy in the following manner:

(a) At the appropriate time each animal received an allogeneic skin graft from a female member of a highly inbred strain of black hooded rats and was at the same time intravenously injected with 1 ml of a 1 per cent suspension of sheep red cells (approximately 10^8 cells). Haemagglutinin titres were estimated on serum obtained 7 days later.

(b) After a month each animal received 10 Lf of tetanus toxoid intraperitoneally and 3 weeks later 10 Lf intravenously. Antibody titres were estimated on serum obtained 7 days after the second injection.

The results were compared with those obtained from appropriate sham- and non-thymectomized control animals.

In another group of animals the same procedure was repeated but on this occasion each animal received a course of ALS prior to grafting and injection of sheep erythrocytes. They were immunized against tetanus toxoid 1 month later. An animal which had been neither operated on nor treated with ALS was included as a control in each group of animals being immunized with tetanus toxoid.

Thymectomy

The manubrium sterni was excised and the bilobed thymus removed by a combination of suction and blunt dissection. The mortality of this procedure was in the region of 5 per cent provided two precautions were taken. Firstly, lymph nodes lying just distal to the thymus may be mistaken for thymic tissue. Their removal usually resulted in uncontrollable haemorrhage. Secondly, the removal of the thymus frequently resulted in a pneumothorax—unilateral or bilateral. The animal will rapidly succumb to this unless air is expelled from the chest by compression and the skin flaps rapidly apposed with a pair of haemostats immediately after the thymectomy. The presence of residual thymic tissue was checked after death by serial histological sections of the mediastinal tissues; residual tissue was present in three animals.

Sham thymectomy. The manubrium sterni was excised. The thymus was pulled up into the wound, dropped back and the wound sutured.

Method of skin grafting

Grafts taken from the ventral surface of the donors and placed on beds prepared in the ventral abdominal skin of the recipients, were held in place with a plastic spray dressing (Nobecutane), covered with gauze and enclosed in a plaster of Paris cylinder. Grafts were kept covered until such time as hair grew or the graft was rejected.

Grafts were first inspected 7 days after operation; daily for the next week and three times a week thereafter. Moistness, oedema or loss of epithelium were considered to be signs of rejection and the first day on which any of these features was evident was considered to be the day of rejection. In long-surviving grafts signs of rejection were seldom clear cut. The day of rejection was considered to be the first day on which the texture or appearance of the graft differed from that of adjacent skin; the usual manifestations were loss of hair, scaling or moistness of the graft surface.

Estimation of haemagglutinin titres

Complement was inactivated by heating serum at 56° for 30 minutes. A volume of 0·2 ml of a 2 per cent solution of sheep erythrocytes was added to 0·2 ml of doubling dilutions of serum in 0·9 per cent NaCl. The dilution in the last well in which the cell pattern differed from the buttons of cells present in the control wells indicated the titre to be recorded. Titrations were done in plastic agglutination trays. An animal was excluded from the investigation if its pre-immunization serum showed detectable antibody.

Tetanus antibody titres

Serum was decomplemented by heating at 56° for 30 minutes. A volume of 0·1 ml of serum was then added to 0·9 ml of isotonic borate buffer pH 8·3, and this solution was adsorbed against 0·1 ml of packed sheep erythrocytes on three successive occasions.

Titrations were done using plastic agglutination plates by a modification of the method of Fulthorpe (1957). A volume of 0·2 ml of formalinized tanned sheep erythrocytes coated with tetanus toxoid was added to 0·5 ml of doubling dilutions of the diluted serum in borate buffer pH 8·3. A similar volume of uncoated tanned cells was added to each one of the control wells. The most reliable end point was that described by Fulthorpe (1957); i.e. a smooth carpet of cells with the faintest ring of more concentrated cells commencing at the periphery. Titres were divided by the titre of a standard antiserum containing 0·7 units of antitoxin/ml (as assayed by Burroughs Wellcome Research Laboratories in the mouse) and titrated without dilution at the same time as the serum being examined. This ratio was the figure finally recorded.

White cell counts

All estimations were done on blood obtained from the tail vein. A volume of 20 mm^3 of blood was added to 0·38 ml of 1 per cent acetic acid and counts were performed in a Neubauer haemocytometer. Differential counts were performed on Leishman stained smears, 200 cells being counted on each occasion. Small and large lymphocytes were counted separately but the figures have been summed and recorded as total lymphocytes in order to avoid discrepancies due to differences in subjective interpretation from time to time.

Anti-lymphocyte serum (ALS)

Mesenteric and thoracic lymph nodes were removed from adult female Sprague-Dawley rats and homogenized in 0·9 per cent NaCl. Each immunizing dose contained $1·2–2·0 \times 10^8$ cells; this was approximately the number of cells obtainable from the mesenteric and thoracic lymph nodes of one rat. Each rabbit received three subcutaneous injections of one immunizing dose at weekly intervals and was bled out 1 week after the last injection. The serum was not heated to destroy the toxic factors described by Terasaki, Esail, Cannon and Longmire (1961), nor were haemagglutinins removed.

Serum was stored at −20°. A batch of serum was considered satisfactory if 4 hours after the intraperitoneal injection of 1 ml per 100 g body weight into an adult female Sprague-Dawley rat (150–200 g) the total lymphocyte count had been at least reduced to 15 per cent of the pre-injection value.

The animals receiving ALS were given 1 ml per 100 g body weight daily for 8 days by intraperitoneal injection. Five days after the last injection they were skin grafted and injected with sheep erythrocytes and a month later they were immunized with tetanus toxoid.

RESULTS

TOTAL LYMPHOCYTE COUNTS

From 120 days after thymectomy the thymectomized animals had total lymphocyte counts which were about 60 per cent of those of sham-thymectomized animals (Fig. 1). The differences were statistically significant in the 120, 180 and 240 days post thymectomy groups. Both thymectomized and sham-thymectomized animals showed a fall in total lymphocyte counts between 150 and 200 days after birth.

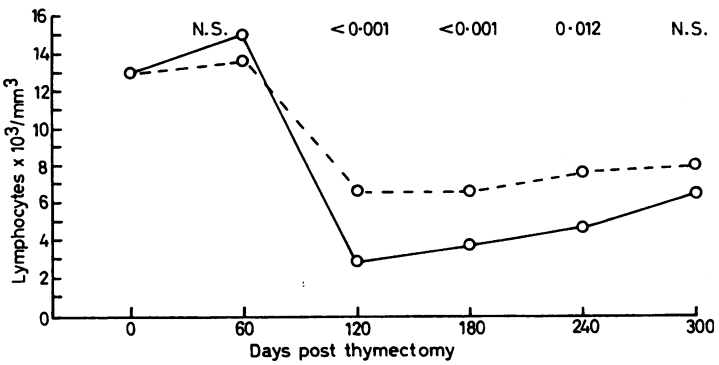

FIG. 1. Total lymphocyte counts of groups of thymectomized (represented by the continuous line) and sham-thymectomized (represented by the broken line) Sprague-Dawley rats operated on when 12 weeks old (150–200 g body weight) and examined at varying intervals after operation. At each time interval, the *P* value for the difference between the counts of thymectomized and sham-thymectomized animals has been calculated on the basis of a Students '*t*' distribution. N.S. = not significant.

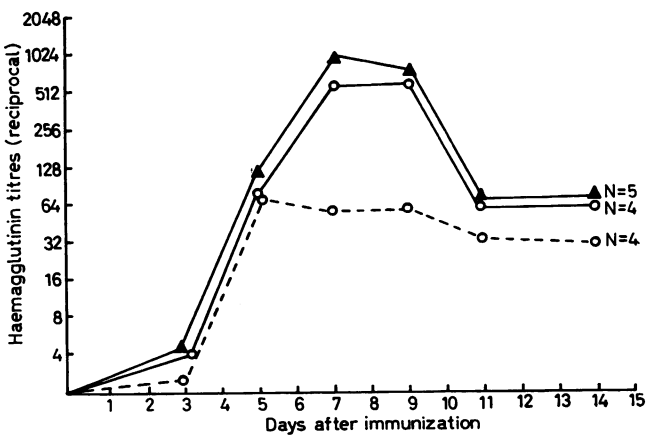

FIG. 2. Serial haemagglutinin titres after the intravenous injection of 1 ml of a 1 per cent solution of sheep erythrocytes. ▲—▲, Non-thymectomized animals; O—O, non-thymectomized animals which have completed a course of ALS 5 days prior to immunization; O - - - O, thymectomized animals which have completed a course of ALS 5 days prior to immunization.

ANTI-SHEEP ERYTHROCYTE HAEMAGGLUTININ TITRES

Both thymectomized and sham-thymectomized animals produced approximately similar titres up to 240 days after thymectomy (Table 1).

There was no significant difference between the titres produced by untreated control animals and control animals given ALS. Each group of thymectomized animals given ALS

TABLE 1

PRODUCTION OF ANTI-SHEEP ERYTHROCYTE HAEMAGGLUTININ BY THYMECTOMIZED, SHAM-THYMECTOMIZED AND BY THYMECTOMIZED AND NON-THYMECTOMIZED RATS GIVEN ALS

Animals	Days after operation	Number	Mean (log_2 titres)	Range (log_2 titres)
Sham-thymectomized animals	0*	25	10·92	6–13
	60	6	11·4	11–12
	120	4	9·7	9–10
	180	4	12·9	10–14
	240	4	11·6	9–12
Thymectomized animals	0	6	10·7	10–12
	60	5	10·8	10–12
	120	7	10·2	9–11
	180	9	10·6	9–14
	240	6	10·3	9–12
Non-thymectomized animals given ALS	0	6	8·43	8–11
Thymectomized animals given ALS	0	6	5·4	3–7
	60	4	4·75	5–6
	120	5	4·8	3–6
	180	4	3·4	0–6
	240	4	4·25	0–6

ALS = Rabbit anti-rat lymphocyte serum. * Animals in this group were not thymectomized.

Separate groups of thymectomized and sham-thymectomized Sprague-Dawley rats operated on when 12 weeks old (150–200 g body weight) and intravenously injected with 1 ml of a 1 per cent solution of sheep erythrocytes at varying intervals after operation. Haemagglutinin titres estimated on serum obtained 7 days later. Animals given ALS received 1 ml per 100 g body weight by intraperitoneal injection daily for 8 days and were challenged with antigen 5 days after the last injection of ALS.

TABLE 2

ALLOGENEIC SKIN GRAFT REJECTION TIMES

Animals	Days after operation	Number	Mean (days)	Range (days)
Sham-thymectomized animals	0*	32	8·3	7–12
	60	6	9·6	9–11
	120	5	9·6	9–12
	180	4	11	11
	240	4	9·5	9–11
Thymectomized animals	0	6	9·7	9–11
	60	5	9·6	8–10
	120	7	21	19–28
	180	8	18·5	15–25
	240	6	21	15–29
Non-thymectomized animals given ALS	0	6	11	9–14
Thymectomized animals given ALS	0	5	29	16–42
	60	4	30·75	21–41
	120	5	20·6	20–23
	180	4	43·75	32–51
	240	4	62	44–112

ALS = Rabbit anti-rat lymphocyte serum. * Animals in this group were not thymectomized.

Separate groups of thymectomized and sham-thymectomized Sprague-Dawley rats operated on when 12 weeks old (150–200 g body weight) and grafted with skin from black hooded rats at varying intervals after operation. Animals given ALS received 1 ml per 100 g body weight by intraperitoneal injection daily for 8 days and were grafted 5 days after the last injection of ALS.

however showed a statistically significant depression of haemagglutinin titres when compared with any one of the other two groups. Values for P calculated on the basis of a Students 't' distribution varied from $<0{\cdot}001$ in the 60 and 120 days post thymectomy groups to approximately 0·04 in the 180 and 240 days post thymectomy groups.

Serial haemagglutinin titre estimations (Fig. 2) indicate that these results were not due to a peak titre having been missed.

SKIN GRAFT SURVIVAL TIMES

From 120 days after thymectomy skin graft rejection times were about twice those shown by sham-thymectomized animals (Table 2). The differences were statistically highly significant. At 120 days P was $<0{\cdot}001$, at 180 days $<0{\cdot}001$ and at 240 days $0{\cdot}001 < P < 0{\cdot}002$.

Graft rejection times were over 25 days in the majority of thymectomized rats given ALS with the exception of the 120 days post thymectomy group of animals. In contrast non-thymectomized animals given ALS did not show an increase in graft survival when compared with untreated animals. Graft survival times were longer in the 180 and 240 days post thymectomy ALS treated groups than in any of the other three groups of thymectomized animals given ALS. The differences were only significant at the 5–10 per cent level.

TABLE 3

TETANUS ANTIBODY TITRES*

Animals	Days after operation	Number	Mean	Range
Sham-thymectomized animals	0†	14	97	20–320
	60	6	60	40–320
	120	5	76	40–160
	180	4	100	80–160
	240	2	160	160
Thymectomized animals	n.d.	n.d.	n.d.	n.d.
	60	5	100	40–160
	120	8	61	10–160
	180	9	67	10–160
	240	4	360	160–640
Non-thymectomized animals given ALS	0	5	88	40–160
Thymectomized animals given ALS	0	4	90	40–160
	60	3	53	40–80
	120	5	100	40–160
	180	5	170	40–320
	240	4	0·31	0–1·25

* Titres expressed as: $\frac{\text{Titre of serum being examined}}{\text{Titre of standard antiserum containing 0·7 units/ml}}$

† Animals in this group were not thymectomized.

ALS = Rabbit anti-rat lymphocyte serum.

n.d. = not done.

Separate groups of thymectomized and sham-thymectomized Sprague-Dawley rats operated on when 12 weeks old (150–200 g body weight) and immunized with tetanus toxoid at varying intervals after operation. At the times indicated in the table each animal received 10 Lf of tetanus toxoid by intraperitoneal injection and 3 weeks later 10 Lf of tetanus toxoid intravenously. Antibody titres were estimated on serum obtained 7 days after the second injection. Animals given ALS received 1 ml per 100 g body weight by intraperitoneal injection daily for 8 days and were challenged with antigen 1 month after the last injection of ALS.

TETANUS ANTIBODY TITRES

All sham-thymectomized animals, all thymectomized animals and all thymectomized animals given ALS and immunized a month later with tetanus toxoid showed approximately similar levels of antibody when tested up to 270 days after thymectomy (Table 3). The one exception was the 240 days post thymectomy group of animals given ALS and immunized a month later with tetanus toxoid ($P < 0{\cdot}001$). Three of the animals in this group did not produce detectable antibody and the fourth a relatively small amount. One incompletely thymectomized animal in this group produced a normal titre of antibody and so did the normal control rat always included in every group of animals being immunized with tetanus toxoid. It is therefore unlikely that this result could have been due to an artefact.

DISCUSSION

The depression of total lymphocyte counts shown by the thymectomized rats in this investigation is similar to the findings of Biering (1960) who showed this phenomenon as early as 65 days after thymectomy. The findings differ from those obtained by Aisenberg and Wilkes (1964), who were unable to demonstrate any fall in total lymphocyte counts 4–6 months after thymectomy. These findings might have been due to the fact that some of their animals were suffering from non-specific pulmonary infections (Aisenberg, 1964, personal communication).

Metcalf (1956) has claimed that a specific lymphocytosis stimulating factor is elaborated by the thymus. Levy, Trainin and Law (1963) were able to restore almost to normal the lymphocyte counts of neonatally thymectomized mice by implanting diffusion chambers containing thymus tissue in these animals. The findings of Osoba and Miller (1963) have not however been as conclusive; these workers were able to restore to normal the lymphocyte counts of only a small proportion of the neonatally thymectomized animals in whom they implanted diffusion chambers containing thymic tissue. The lymphopenia noted by us could therefore have been, in part, due to the absence of a humoral lymphocytosis stimulating factor.

The thymus has been shown to display intense lymphopoietic activity (Andreasen and Christensen, 1949) and Kindred (1942) has suggested that cell death in the thymus is no more than 15 per cent of cell production. The removal of a large mass of lymphocyte-producing tissue could therefore have been another factor responsible for the development of a lymphopenia by these animals.

The difference in the lymphocyte counts of thymectomized and sham-thymectomized animals was not statistically significant 300 days after thymectomy. This might have been due to the small number of animals available in these two groups. Another possibility is that thymic lymphopoietic activity declines with the passage of time with a corresponding increase in extra-thymic lymphopoiesis.

Both thymectomized and sham-thymectomized animals showed a fall in total lymphocyte counts between 150 and 200 days after birth. This phenomenon has been investigated in unoperated rats of the strain used in this investigation and it would appear to be normal in these animals.

As judged by its response to sheep erythrocytes and tetanus toxoid, the rat which has been thymectomized in adult life shows no decrease in its ability to produce circulating antibody; even when tested as long as 270 days after thymectomy. Aisenberg and Wilkes

(1964) have shown that the rat which has been thymectomized in adult life is also capable of mediating a normal humoral antibody response to both bovine serum albumin and human γ-globulin when tested 4–6 months after thymectomy. If the thymectomized animals in this investigation had been tested later it is possible that a different result might have been obtained. A view widely held at present is that there are two populations of small lymphocytes; one long lived and the other relatively short lived. The work of Hamilton (1956) suggests that the former have life cycles upwards of 250 days.

From 120 days post thymectomy onwards the thymectomized rats in this investigation show a modest but statistically significant decrease in their ability to reject allogeneic skin grafts. Aisenberg and Wilkes (1964) have reported that rats thymectomized in adult life also show a decrease in their ability to mediate dermal delayed hypersensitivity reactions to bovine serum albumin when immunized 4–6 months after thymectomy. It is difficult to explain satisfactorily the variable depression of the immune response shown by the rat thymectomized in adult life.

The ALS used in this investigation was unheated and the haemagglutinins were not absorbed. Sacks, Fillipone and Hume (1964) have shown that neither procedure affects the lymphocytotoxic properties of ALS.

An interval of 5 days was allowed to elapse between the last dose of serum and challenge with antigen. One hoped that the production of new immunologically competent cells during this interval might enable the immune response of the non-thymectomized animals to be as near normal as possible when they were challenged with antigen. This was done in an attempt to establish a clear cut difference between the immune responses of thymectomized and non-thymectomized animals given ALS. The results obtained suggest that the thymus does continue to have an immunological function in the adult rat.

Graft rejection times in the 180 and 240 days post-thymectomy groups given ALS were longer than those in any of the other three groups of ALS treated animals. In addition the 240 days post-thymectomy group given ALS and tested a month later with tetanus toxoid failed to produce any significant quantities of tetanus antibody.

It is probable that, between the different groups of treated animals, there was no significant variation in the number of cells inactivated by the lymphocytotoxic action of ALS. If this is so the experimental results suggest that with the passage of time there is a decrease in the number of immunologically competent lymphocytes possessed by an animal thymectomized in adult life; skin graft survival times lengthened as the interval between thymectomy and the administration of serum increased.

The results also suggest that a number of cells sufficient to proliferate and produce a detectable antibody response had escaped the lymphocytotoxic action of ALS in all but one group of thymectomized animals; the 240 days post-thymectomy group. In the introduction it was postulated that an adult animal would become incapable of producing new immunologically competent cells once it had been thymectomized. The failure of this one group of animals to produce any significant quantity of antibody when given tetanus toxoid a month after the administration of ALS supports this postulate.

Moreover, the normal response to tetanus toxoid shown by all the other groups of ALS treated animals suggests that immunologically competent cells present at the time of thymectomy remain capable of proliferating and producing antibody when antigenically stimulated—if it is accepted that some cells had escaped the lymphocytotoxic action of ALS in each of the treated animals.

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